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14. ABSTRACT A significant proportion of patients with neurofibromatosis type I (NF-1) will develop benign neurofibromas in their peripheral nerves that will progress to malignant tumors that are called malignant peripheral nerve sheath tumors or MPNST. These tumors grow progressively and if they cannot be completely removed surgically, they are eventually fatal. Radiation provides some benefit but is mostly ineffective and there are no proven chemotherapy type drugs or medicines that will prevent the progressive growth of these tumors. We have been working with genetically engineered human herpes simplex virus (HSV) as a means of treating nervous system tumors. We have genetically modified these viruses to make them safe and unable to grow in normal cells, but they will grow in tumor cells and will eventually cause the tumor cells to die, a process called oncolysis (tumor lysis). In addition, we have inserted several different kinds of genes in these viruses that allow them to overcome different mechanisms that tumor cells have to prevent virus growth.  Our objective is to determine whether or not MPNST cells can be infected and killed by our oHSVs. We will test our panel of viruses against a panel of mouse and human MPNST cells that are maintained in tissue culture. We will examine the molecular changes in this large panel of tumor cells (tumor genotype) to determine how each virus is able to overcome each tumor's anti-virus defenses. We believe that all possible anti-virus defense mechanisms would be represented in our panel of both mouse and human MPNSTs and that we will be able to match the most effective oHSV engineered to overcome that mechanism used to block the virus.				
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## Table of Contents

	<u>Page</u>
<b>1. Introduction .....</b>	<b>1</b>
<b>2. Keywords .....</b>	<b>1</b>
<b>3. Overall Project Summary .....</b>	<b>1</b>
<b>4. Key Research Accomplishments .....</b>	<b>8</b>
<b>5. Conclusion .....</b>	<b>8</b>
<b>6. Reportable Outcomes .....</b>	<b>10</b>
<b>7. References .....</b>	<b>10</b>
<b>8. Appendices</b>	
i. <b>Poster</b>	
ii. <b>Supporting Data</b>	
iii. <b>Publication</b>	

We are pleased to provide an update of our studies involving the use of oncolytic herpes simplex virus type-1 (oHSVs) as a therapeutic agent for MPNSTs. Our most recent year of DOD funding has yielded significant results including a peer-reviewed publication describing the limitations of HSV entry-receptor expression in MPNSTs as well as uncovering a promising distinction between oHSV permissive and resistant phenotypes related to the capacity of resistant cell lines to activate anti-viral signaling pathways.

**Keywords:** MPNST, neurofibromatosis, oncolytic virus, HSV-1, IL-12

In the first year of research, we reported general observations regarding the sensitivity of human and murine MPNST cell lines to a panel of genetically-attenuated oncolytic HSVs using *in vitro* assays. We observed that there existed two general phenotypes: those cell lines in which infection with oHSVs yielded a productive infection (permissive phenotype) and those in which the infection yielded poor evidence of viral productivity (resistant phenotype). A major objective of our research has been to identify the distinguishing features between the permissive and resistant phenotypes. Knowledge of these features will allow us to identify which viruses may have the greatest promise in a clinical setting and inform the future direction of our research so that we might develop strategies to overcome oHSV resistance. We provide in the following report our progress in the pursuit of this goal.

Our goals for this grant are summarized below and include:

- 1) To determine the molecular basis for the sporadic susceptibility or resistance to infection of MPNST cells to genetically engineered, oncolytic herpes simplex viruses (oHSVs) in our repository;
- 2) To examine inherent mechanisms expressed in MPNSTs that inhibit the replication of oHSVs and abrogate the ability of these viruses to kill infected cells and spread to neighboring tumor cells; and
- 3) To test the relative ability of our oHSVs to produce an anti-tumor effect alone and if this anti-tumor effect can be significantly enhanced by a low dose of radiation administered to the tumor.

Thus far our work has focused upon the first 2 milestones listed, and a modification of the third milestone. With respect to the third milestone, we have made significant progress investigating the role of the JAK/STAT1 signaling pathway in oHSV resistance in place of the hypothesis regarding the p38 and MEK/ERK signaling pathways. We have suggested in the following report that the JAK/STAT1 signaling pathway be further examined in place of the originally proposed p38 and MEK/ERK pathways. Preliminary investigation into p38 and MEK/ERK pathways did not suggest that further investigation would be fruitful. We are also recommending that our animal studies described in the fourth milestone further include further assessment of combined radiation and oHSV administration in the context of cell lines which can and cannot activate STAT1. Our progress is summarized below and has been organized within the 4 major milestones listed in our Statement of work and addressed with regard to each of the Tasks and SubTasks.

**Milestone 1:** We will identify at least 2 oHSV-sensitive and 2 oHSV-resistant MPNST cell lines by completing the *in vitro* characterization of both human and mouse MPNST cell lines with respect to oHSV infection and killing. We have preliminary analysis of 2 human and two murine MPNST lines. They range from sensitive to resistant to oHSV infection and killing. This milestone will provide the prototypic MPNST cell lines that will be studied more extensively in all three aims.

**Milestone 2:** We will characterize each of the 9 human MPNST cell line and at least 18 of the 100+ mouse MPNST cell lines with regard to expression of HSV entry molecules expressed on the cell surface. This milestone will enable us to determine whether prevention of entry by down-regulation of appropriate receptors is the reason for oHSV resistance and, if so, whether we should define alternative receptors to which new oHSVs could be targeted.

**Milestone 3:** We will characterize the replication of oHSVs in each of the oHSV-sensitive and oHSV resistant MPNST cells identified in Milestone 1 by FACS and by titering virus at regular post-infection intervals. Within this context, we will establish the extent to which replication is enhanced in infected MPNST cells by oHSVs engineered to express proteins that directly promote virus replication. This milestone will allow us to select either the HCMV IRS-1 or the constitutively activated MEK gene as the most appropriate insert to overcome replication resistance.

**Milestone 4:** We will determine which of the oHSVs identified as “effective” in the first two aims of this proposal actually produce the expected anti-MPNST effect in oHSV-sensitive and oHSV-resistant tumors of human or mouse origin placed orthotopically in the appropriate strain of mouse (see below). Efficacy alone or in combination with enhancing adjunctive therapies will be defined. This milestone will serve to validate (or refute) the process for selection of effective oHSVs that could be advanced to clinical trials in patients with MPNSTs and identify which modality is most likely to have an impact on the natural history of this disease.

**Task 1: Characterize the *in vitro* sensitivity of a panel of human and mouse MPNST cell lines to a panel of available oncolytic HSVs.**

SubTask 1a. Using methods that we have described in Preliminary Findings in the Proposal, we will complete the screening of all 9 human MPNST cell lines and at least 18 of the 100+ mouse MPNST cell lines using FACS for detection of expression and modulation of HSV entry molecules (nectin-1, nectin-2) and alternative entry molecules (HVEM, IL13R $\alpha$ 2, uPAR, Her2/neu) recognized by fluorochrome-labeled antibodies.

Status: Due to contamination of a number of the MPNST cell lines with mycoplasma which was discussed in the previous report, we have changed the number of cell lines for all subsequent studies to 8 human and 14 mouse MPNST cell lines that have been confirmed to be free of mycoplasma.

For a complete description of methodology used in Sub-Task 1a, we refer the reader to the attached publication (Jackson, *et al.*). In summary, all human MPNST cell lines were found to express detectable levels of nectin-1, the major HSV-1 entry receptor by FACS analysis (Publication Figure 1f). One cell line expressed significant levels of the alternative entry receptor HVEM (Publication Supplemental Figure 1d). We overexpressed nectin-1 in oHSV resistant cell lines to determine the effect of increased entry receptor expression on the productive capacity of each cell for oHSV replication and cell-to-cell spread. This did not result in a substantial improvement for the oHSVs by *in vitro* or *in vivo* measurements (Publication Figures 2 and 3) but did yield a benefit to the wild-type virus by *in vitro* assays (Publication Figure 2 and Publication Supplemental Figure 4). We therefore conclude that entry receptor expression is not the primary mechanism of resistance to our attenuated oHSVs. Furthermore, basal levels of nectin-1 expression in MPNST cell lines appear sufficient to mediate entry for wild-type HSV-1 and by extension oHSVs.

Although we did not conduct these experiments in mouse cell lines, the data from human cell lines indicates that the means of resistance to oHSVs lies elsewhere and that entry receptor expression is sufficient, at least in MPNST-derived cell lines. We therefore do not plan to expend any additional resources into the investigation of HSV entry receptors.

SubTask 1b. Using methods that we have described in Preliminary Findings in the Proposal, we will complete the screening of the human and mouse MPNST cell lines using oHSVs that express eGFP for detection of infection and cell death using FACS.

Status: We have completed the screening of the 8 human and 14 mouse MPNST cell lines using representative viruses with eGFP expression. In this assay we infected each cell line at a multiplicity of infection (MOI) of 0.1 and then subjected the cells to FACS detection of viral GFP at 48 hours post infection (hpi). We have simultaneously measured the absolute cell killing of each virus using FACS counting beads. Both second generation viruses C154 and M201 demonstrated significantly higher GFP positive cells (Figure 1a) and reduction in relative cell counts (Figure 1b) as compared to C101. As expected the wild-type virus also demonstrated these increases (Figure 1a-b). We expect that cell lines which support viral productivity will show an increased number of cells positive for GFP and a simultaneous decrease in

the number of cells as compared to an uninfected control. As demonstrated in Figure 1 c-e, the percentage of cells expressing viral GFP is significantly correlated with the relative decrease in cell numbers for viruses C101 ( $\Delta\gamma_{134.5}$ ), C154 ( $\Delta\gamma_{134.5}$ , IRS-1), and M201 ( $\Delta\gamma_{134.5}$ , IL-12) but not for the representative wild-type virus M2001 (Figure 1 f). The fact wild-type HSV-1 infected have low cell counts but no correlative in GFP expression is likely due to the rapid course of infection for the wild-type HSV-1 compared to  $\Delta\gamma_{134.5}$  oHSVs, such that by 48 hpi wild-type infected cells are likely no longer actively expressing GFP due to cell death in certain cell lines.

This data suggests that this FACS based assay is a valid measurement of oHSV productivity. It effectively divides oHSV resistant cells (low number of GFP positive cells, high relative cell counts) from oHSV permissive cell lines (high number of GFP positive cells, low relative cell counts). This experiment also confirms that both C154 and M201 (and by extension the non-GFP expressing viruses C134 and M002) are more effective than a first-generation  $\Delta\gamma_{134.5}$  oHSV (C101, based on R3616). All MPNST cell lines are susceptible to wild-type HSV-1 (M2001).

SubTask 1c. Screen each of the 9 human and 18 mouse MPNST cell lines for sensitivity to infection and killing by clinical candidate viruses G207, NV1020, M032 and C134 using classical virology techniques to measure cytopathic effect on monolayers, single step and multi-step replication assays.

Status: This task has been previously addressed (Figures 2 and 3). In summary, there is variation in the ability of each cell line to support replication of oHSVs. All cell lines support higher levels of wild-type HSV-1 (M2001). However, some cell lines (i.e. S462-luc) support replication of all oHSVs including fully attenuated first-generation oHSVs (C101, G207) and second-generation oHSVs (M002, M032, C134), while other cell lines tend to only support replication of the wild-type virus and viruses such as C134 which are engineered to counteract the anti-viral response. This is concordant with other assays and gives support to the hypothesis that the loss of the  $\gamma_{134.5}$  gene is irrelevant in some cell lines but its function may be necessary in others to support a productive infection.

SubTask 1d. Correlate the data in the described experiments to identify oHSV-sensitive and oHSV-resistant MPNSTs and select 2 of each to study in the subsequent experiments.

Status: These results were completed in year 1 when we chose representative HSV sensitive and resistant human and murine cell lines based upon viral replication (Subtasks 1a-c). For the correlation of receptor expression (nectin-1) with viral titers, we refer the reader to the Publication Figure 1 a-e in the attached manuscript. For correlation with GFP and cell count data please refer to SubTask 1b.

Human HSV Sensitive cell lines: (S462-luc and NMS2-PC)

Human HSV-Resistant cell lines (T265-luc and STS26T-luc)

Murine HSV Sensitive cell lines (A18 and 231 Trig)

Murine HSV Resistant cell lines (A382 and A202)

We will use the cell lines identified above to further test hypotheses regarding signaling pathways described in Aim 2.

SubTask 1e. Correlate the expression of alternative molecules on oHSV-resistant MPNSTs with the potential to engineer oHSVs that can utilize these receptors to enter cells that resist HSV entry.

Status: We have published the results of this correlation in the attached article (Jackson *et al.*) (Figure1 a-e). In summary, no oHSV (G207, C101, R7020, M002, M032, or

C134) had a significant correlation with the capacity to support viral replication. Although not statistically significant, the second generation virus C134 showed a trend toward increased replication with increased nectin-1 expression. The wild-type virus did in fact show a strong and significant correlation with viral titers and entry-receptor expression. We refer the reader to the attached article and to the comments in SubTask 1a for further detail.

**Task 2: Establish the most effective means of enhancing virus replication by modifying a HSV-resistant phenotype.**

**SubTask 2a.** In Aim 2, we will test two different engineering solutions to enhance the expression of HSV “late genes” in both oHSV-sensitive and –resistant MPNST cell lines. We will use a combination of classical virology methods (plaque-titering at 24hr-intervals boost infection; single-step & multi-step replication assays) and FACS monitoring the extent and time course of oHSV infection based on expression of eGFP and other fluorescent markers by FACS assays

**Status:** We have shown that viruses capable of evading IFN mediated antiviral mechanisms are superior to those containing uncompensated  $\gamma$ 134.5 deletions in MPSNT lines suggesting that IFN activation is the principal impediment of oHSV replication and spread in the MPNST cells. We are in the process of testing this hypothesis and the IFN mediated mechanisms limiting viral replication and spread.

Our studies to date have established that all MPNST cell lines are susceptible to the wild-type virus, however only certain cell lines are permissive to  $\Delta\gamma$ 134.5 oHSVs. This has led us to hypothesize that the functions of the  $\gamma$ 134.5 gene product ICP34.5 may be necessary in resistant cell lines but dispensable in permissive cell lines. We will discuss briefly the known functions of ICP34.5 to establish our recent work which has yielded significant results and a more promising line of research than what was originally proposed.

The  $\gamma$ 134.5 gene product ICP34.5 has several documented functions. ICP34.5 recruits the protein phosphatase 1-alpha (PP1 $\alpha$ ) to protein kinase R (PKR) in order to reverse the phosphorylation of PKR. PKR is normally phosphorylated following detection of double-stranded RNA, such as that produced upon viral transcription. This phosphorylation event activates PKR to phosphorylate its target eukaryotic translation initiation factor 2A (eIF2a). Phosphorylation of eIF2a inhibits translation of messenger transcripts including those produced by the virus. The dephosphorylation of PKR by ICP34.5 therefore reverses this translational arrest and permits viral protein translation to continue. We have demonstrated that both permissive and resistant MPNST cell lines phosphorylate eIF2a in response to R3616, a representative  $\Delta\gamma$ 134.5 oHSV (Figure 4). Furthermore, PKR is phosphorylated in the majority of human MPNST cell lines including the previously established permissive cell lines (e.g. S462-luc and NMS2PC). A mouse compatible p-PKR antibody has not yet been acquired. Therefore the lack of PKR/eIF2a phosphorylation in permissive cell lines does not explain the difference observed between the permissive and resistant cell lines.

A second known function of ICP34.5 is the inhibition of TANK binding kinase-1 (TBK-1) activation<sup>1</sup>. TBK-1 is a downstream target of pattern recognition receptors (PRRs) which detect pathogen associated molecular patterns (PAMPs, e.g. viral dsRNA). The normal consequence of TBK-1 activation is the phosphorylation of interferon regulator factor-3 (IRF-3) and the subsequent binding of IRF-3 to interferon promoter elements which increases transcription of Type-I interferons (IFNs) which include the cytokines IFN- $\alpha$  and IFN- $\beta$ <sup>2</sup>. Autocrine and paracrine interaction of extracellular Type-I IFN with its cognate receptor stimulates Janus kinases (JAK) to phosphorylate the signal transducer and activator of transcription-1 and 2 (STAT1/2). STAT dimers then enter the nucleus and promote transcription of a host of interferon

stimulated genes (ISGs)<sup>3</sup>. These ISGs are primarily involved in amplifying and promoting viral resistance<sup>3</sup>. Therefore, ICP34.5 inhibition of TBK-1 results in the loss of Type-I interferon expression and thus subsequent activation of the associated STATs and target ISGs.

To test the hypothesis that permissive cell lines did not respond to  $\Delta\gamma$ 34.5 oHSV infection by activating STAT1, we performed western blot analysis of mock or R3616 infected cell lysates to look at the phosphorylation of STAT1. Cells were infected at an MOI of 1 and lysates collected at 6 hpi. The timepoint 6 hpi was confirmed in our assays to represent the peak of STAT1 phosphorylation with subsequent reduction in phosphorylation at subsequent timepoints. STAT1 phosphorylation was observed in 2 of 8 human MPNST cell lines (STS26T-luc and 88-14-luc) and 7 of 14 mouse cell lines (Figure 5). STAT1 was expressed in all cell lines (Figure 5) and all human MPNST cell lines responded to exogenously applied IFN- $\beta$  with STAT1 phosphorylation (Figure 6) (murine cell lines are in the process of being tested) indicating that all (human) cell lines have functional IFN receptors and JAKs. We then separated the quantitative data of viral productivity acquired from our previous experiments into pSTAT1+ and pSTAT1- groups and tested whether there was a statistically significant difference between the groups. We found significant inverse associations ( $P<0.05$ ) between the ability of a cell line to activate STAT1 and the capacity to support productive viral infection. The percentage of cells positive for viral GFP at 48 hpi was significantly increased for C101, C154, and M201 in cells which did not activate STAT1 (Figure 7 A-C). In addition there was a significant decrease in the relative cell counts for C154 and M201 in cell lines which did not activate STAT1 (Figure 7 F-G). There was no difference observed between the STAT1 phosphorylation status with the wild-type virus as both pSTAT1+ and pSTAT1- cell lines were statistically indistinguishable by percentage GFP positive and relative cell loss measurements (Figure 7 D and H). Titers of R3616 infected cells (MOI=1, 24 hpi) were also significantly increased in pSTAT1- cell lines (Figure 8).

These findings have led us to conclude that all  $\Delta\gamma$ 34.5 oHSVs, including the second-generation M002 and C134 viruses, are significantly inhibited in cell lines which are competent in their ability to respond to HSV infection by activating the antiviral cascade involving STAT1. The association of pSTAT1 with resistance leads us to question whether pSTAT1 mediates this resistance or whether STAT1 phosphorylation occurs as a consequence of, or in parallel with the actual mediators of resistance. We are currently testing the first hypothesis, that pSTAT1 mediates this resistance, by stably expressing a dominant-negative STAT1 (dnSTAT1) which will result in a loss of function for STAT1 transcriptional activation. We will further test the role of Type I IFNs in inducing pSTAT1 by using neutralizing antibodies to IFN $\alpha$  and IFN $\beta$  and observe the effect upon  $\Delta\gamma$ 34.5 oHSV infection in resistant cells. We will perform the reciprocal experiment in permissive cell lines by observing the extent to which exogenously applied IFN $\beta$  inhibits productive  $\Delta\gamma$ 34.5 oHSV infection in normally permissive MPNST cell lines.

Finally, cell lines which are capable of responding to infection with oHSVs by activating the IFN/STAT1 cascade may be primed to resist the virus prior to infection. We have observed that basal levels of interferon stimulated genes (ISGs), the transcriptional targets of pSTAT1, are elevated at baseline in pSTAT1+ human MPNSTs (Figure 9). Both pSTAT1+ cell lines STS26T-luc and 88-14-luc show elevated expression of the ISGs myxovirus resistance-1 (MX1) and interferon-induced protein with tetratricopeptide repeats 3 (IFIT3) as compared to pSTAT1- cell lines. This may indicate aberrant low-level expression of IFN in these cell lines which elevates the endogenous levels of ISGs that mediate resistance to oHSV and primes them for oHSV resistance. We will test this by exposing these cell lines to neutralizing IFN antibodies and observe any transient decrease in ISG expression.

Basal expression of ISGs will also be tested in our dominant-negative STAT1 cell lines and compared to the parent cell lines.

SubTask 2b. Determine the ability of HSV-mediated expression of constitutively activated-MAP kinase kinase (MEK) will result in an increase in HSV late gene expression, higher HSV particle production and cytotoxicity.

Status: Preliminary examination of MEK activity assessed by immunoblots of phosphorylated ERK 1/2 (the established target of MEK) indicated that MEK is active at basal levels as well as following viral infection in many MPNST cell lines (Figure 10). Most significantly in oHSV resistant cell lines T265-luc and STS26T-luc demonstrate basal and sustained ERK 1/2 phosphorylation suggesting that activated MEK is not sufficient to impart nor does it correlate with an oHSV permissive phenotype. For example basal activity of MEK is not apparent in the permissive cell line YST-1 and yet this cell line yields consistently higher titers of virus as compared to the resistant cell lines.

Due to the findings regarding the activation of the STAT1 pathway discussed in SubTask 2a, we believe that our efforts will be more fruitful by further investigating the STAT1 signaling pathway as opposed to the MEK/ERK pathway which has not shown promise in preliminary work. Thus, the focus of this subtask has been changed as a result of data generated during the performance of our work related to this grant.

SubTask 2c. Determine the ability of HSV-mediated expression of a human cytomegalovirus gene, IRS-1, that promotes late gene expression in CMV, to increase oHSV late gene expression, higher HSV particle production and MPNST cytotoxicity.

Status: We have determined that the IRS-1 expressing oHSV C134 (and the eGFP variant C154) significantly improves the productive capacity of  $\Delta\gamma$ 134.5 oHSVs. This is evident by an increase in viral titer (Publication Supplementary Figure 1b) as well as by increased cell-to-cell spread (Figures 1a and Publication Figure 3 d-e) and a reduction in relative cell counts as compared to the unmodified  $\Delta\gamma$ 134.5 oHSVs C101 and R3616 (Figure 1b). Although C134 approaches the replicative capacity, spread, and cell killing that the wild-type virus does in a number of cell lines, there are still cell lines which resist this virus. This indicates that the expression of IRS-1 and consequent inhibition of PKR activation may not be sufficient to reverse the  $\Delta\gamma$ 134.5 oHSV resistant phenotype. We further show in SubTask 2a that cell lines which are capable of activating the antiviral signaling pathway mediated by STAT1 have significantly diminished capacity to support the productive infection of  $\Delta\gamma$ 134.5 oHSVs including C134.

SubTask 2d. Examine the impact of p38MAPK activation in MPNST tumors to have a positive or negative impact on the ability of these engineered oHSVs to display greater replication and oncolysis.

Status: Preliminary western blots suggest that there is p38 activation in both resistant and sensitive cell lines (Figure 10). These studies were being actively pursued as we entered year 3.

Similar to our discussion in SubTask 2b, due to the findings regarding the activation of the STAT1 pathway discussed in SubTask 2a, we believe that our efforts will be more fruitful by further investigating the STAT1 signaling pathway as opposed to the p38 pathway which has not shown promise in preliminary work.

SubTask 2e. Correlate and compare the data sets obtained from the studies in oHSV-sensitive and –resistant MPNSTs using the caMEK viruses (R2660, R2636) and the IRS-1 viruses (C134, C154).

See explanation in SubTask 2d. While we do not anticipate further exploring the p38 or MEK/ERK pathways, we will have correlated the STAT1 status with the panel of oHSVs as described in SubTask 2a.

SubTask 2f. Select the most appropriate (set of) oHSV virus(es) to advance to preclinical *in vivo* studies with human and mouse MPNSTs.

Status: IN PROGRESS. For the human cell lines, we have discovered that the IFN evasion virus holds a distinct advantage (spread by GFP assay) over first generation viruses and second generation viruses incapable of IFN evasion (M201, M002, M032). Because the cytokine expressing virus would provide less advantage as a therapeutic in the athymic models, we chose to limit our analysis in preliminary studies to C134 and its parent virus C101. In our later studies we have also begun to investigate the viruses for which clinical grade product is available. Now that we have identified that the human tumor experience is limited in the *in vivo* model, we are beginning to investigate the syngeneic models but have not begun these implantations. For the syngeneic studies, we hypothesize (as stated in our previous progress report) that in addition to the C134 based virus, the M032 virus will also be an especially attractive virus to test because of its ability to enhance an immune-mediated anti-tumor response. The xenogeneic models with human tumors in immunocompromised mice have permitted evaluation of the 3 clinical candidate viruses as well as differences in C101 and C154. We are now initiating studies in the syngeneic mouse models and will assess anti-tumor efficacy and how the host immune response impacts efficacy. These studies are the basis of Milestone 4.

**Task 3: Validate the ability of selected oHSV to produce an oncolytic anti-MPNST effect in established tumors in mouse models and quantify the capacity of a low dose of radiation to enhance this anti-tumor effect.**

These studies have not yet commenced. We are currently waiting to conduct these studies using tumors with defined STAT1 activation patterns. The literature indicates that the ability of a tumor to activate the STAT1 signaling cascade is directly related to radioresistance<sup>4</sup>. We have therefore sought to characterize our *in vivo* radiation experiments in the context of STAT1 competent and dnSTAT1 cell lines. We are fortunate to have both human and murine cell lines which show differential activation of STAT1 in response to oHSV and therefore plan to advance the *in vivo* studies using these lines. We have already demonstrated that the pSTAT1+ cell line STS26T-luc cell line establishes tumors *in vivo* and should provide a good model to test the effects of a dnSTAT1 with oHSV with and without radiation.

SubTask 3a. Tumor cells growing *in vivo* often display significant biologic differences from those growing *in vitro*. The first subtask will be to establish a baseline of the ability of oHSVs to infect and kill human or mouse MPNST cell lines transplanted into appropriate host mouse strains. The ability of generic  $\Delta\gamma$ 34.5 HSV (G207, NV1070) to produce an antitumor effect as observed in Task 1c *in vitro* will be determined by direct injection of bioluminescence-enabled human or mouse MPNSTs placed in an orthotopic location (sciatic nerve). Both oHSV-sensitive and oHSV-resistant MPNSTs will be compared.

Status: We have completed training of sciatic nerve tumor grafting and plan to advance *in vivo* studies within the next year.

SubTask 3b. Compare the abilities of selected oHSVs (e.g., M002, C134, R2660, etc) from previous studies to produce an enhanced anti-MPNST effect compared to that of the generic viruses. Oncolysis of orthotopically-placed oHSV-sensitive and oHSV-resistant MPNSTs will be compared.

Status: We have completed training of sciatic nerve tumor grafting and plan to advance *in vivo* studies within the next year.

SubTask 3c. Determine whether or not a single low dose of radiation (2-5Gy) delivered to the tumor within 24 hrs of injection of selected oHSVs enhances the replication and spread of the virus yielding an enhanced anti-MPNST effect. Irradiation has a more pronounced and sometimes paradoxical effect in vivo than it does in vitro and thus, irradiation effects will not be explored in vitro.

Status: We have completed training of sciatic nerve tumor grafting and plan to advance in vivo studies within the next year.

SubTask 3d. Compare and correlate the findings from these sub-tasks to select the most likely combination of oHSV and adjunctive therapy that will be most effective oncolytic, anti-MPNST modality for human or mouse MPNSTs transplanted orthotopically and test this combination in the P<sub>0</sub>-GGFβ3 x Elux mouse against MPNST tumors that arise sporadically and spontaneously.

Status: We have not obtained sufficient data to be able to complete this subtask.

SubTask 3e. Review the entire data set to design studies that will be able to validate the selected oHSV with or without adjunctive therapy that can be advanced to a Phase I/II clinical trial to test the safety, identify unanticipated toxicities and establish preliminary evidence of efficacy in patients with MPNST.

Status: We have not obtained sufficient data to be able to complete this subtask.

## Key Research Accomplishments

- Peer-reviewed publication documenting our studies of entry receptor expression in the context of different viral genotypes
- Nectin-1 is expressed in all of our MPNST cell lines; Bayesian analysis suggests that this is the probable state for MPNST tumors in general
- HVEM expression is present in at least one of our cell lines, however its limited or non-existent expression in the remaining cell lines would not suggest its role as a significant player in HSV entry in MPNSTs
- Even low levels of Nectin-1 expression, as determinable by current techniques, appear sufficient to permit viral entry and subsequent replication in our MPNST cell lines
- All MPNST cell lines are competent in their ability to phosphorylate eIF2a in response to infection with oHSV
- No correlation exists between the PKR/eIF2a activation status and permissive/resistant phenotypes
- The ability of a cell line to activate the antiviral cascade involving STAT1 in response to oHSV infection is significantly and negatively correlated with the productive capacity of □<sub>134.5</sub>-deleted viruses, including second-generation viruses
- Wild-type HSV-1 demonstrates productive infection in all cell lines regardless of their capacity to activate STAT1
- Cell lines which demonstrate STAT1 activation also have greater expression of ISGs prior to infection
- Multistep assays using □<sub>134.5</sub>-deleted viruses demonstrate significant correlation between the ability of the virus to spread from cell-to-cell and the relative loss of cells.

## Conclusions

As a first approach to distinguish between permissive and resistant phenotypes, we hypothesized that the expression of the entry receptors which the virus uses to gain access into the cell was limited in cells which were resistant to oHSV. We tested this hypothesis by overexpressing the major HSV-1 entry receptor nectin-1 in cells which we had previously identified as resistant. By several measures, this increase in nectin-1 did little to improve the productive capacity of our oHSVs and did not reproduce the

permissive phenotype observed in other cell lines. We have therefore concluded that differences in HSV-1 entry receptor expression do not explain the disparate phenotypes observed in MPNSTs.

These conclusions are counter to those reported for oHSVs in other cancer types. These other studies demonstrate that the level of entry receptor expression is directly related to oncolytic efficacy. One major difference between these studies is the use of oHSVs which contain a functional copy of the HSV neurovirulence gene  $\gamma$ 134.5. The viruses used in our lab are all based upon the deletion of both copies of  $\gamma$ 134.5. Indeed, these viruses did not demonstrate a correlation with entry receptor expression nor did they substantially benefit from increased entry receptor expression. However, in our studies the wild-type virus did show dramatic benefit and showed significant correlation with entry receptor expression. We have also shown that all MPNST cell lines are susceptible to productive infection by the wild-type virus. This has led us to conclude that entry receptor expression is not the primary mode of resistance.

The results regarding the involvement of the STAT1 signaling pathway have provided us with a promising new approach to determine the source of resistance to our oHSVs. Although we have not yet performed the studies which look into the function role of STAT1 activation (using a dnSTAT1), early data indicates that there is a significant association with a cell's ability to activate this pathway and the outcome of the infection. Interestingly, this association holds for the second-generation  $\Delta$  $\gamma$ 134.5 oHSVs (C134 and M201) despite the fact that these viruses perform much better than the first-generation  $\Delta$  $\gamma$ 134.5 oHSV C101. The virus C134 expresses the chimeric transgene HCMV IRS-1 which has been shown to inhibit the activation of PKR thereby preventing translational arrest. This would presumably compensate for one function of ICP34.5. The STAT1 data however suggests that prevention of PKR activation may not be sufficient to allow a productive infection in cells which are STAT1 responsive. This observation may be due to the other roles that ICP34.5 provides during infection, including the prevention of TBK-1 induced expression of IFN and the subsequent activation of STAT1. We are excited to further pursue these studies as they have not yet been explored in the context of oHSV virotherapy. Furthermore, pharmacologic modulators exist for the STAT1 signaling cascade which may suggest a potential avenue for adjuvant therapies which overcome resistance attributable to STAT1 activation.

## **Recommend Changes in Future Work**

In light of the findings regarding the IFN/STAT1 pathway described in SubTask 2a, we recommend the work in the remaining NCE year focus on the relevance of this pathway to oHSV resistance and permissive phenotypes. We believe this is a more fruitful approach in contrast to the p38 and MEK/ERK pathways, based upon our findings which demonstrate statistically significant inverse association with STAT1 activation and the productive capacity of  $\Delta$  $\gamma$ 134.5 oHSVs. In addition, the existence of pharmacologic modulators of STAT1 activation will allow us to test whether use of these drugs *in vitro* and *in vivo* settings will assist in overcoming the resistance associated with the activation of this pathway. Both ruxolitinib<sup>5, 6</sup> (a JAK1/2 inhibitor) and fludarabine<sup>7, 8</sup> (a general STAT1 inhibitor) are FDA approved drugs and have documented effects (see references) which benefit other oncolytic virotherapies but have not yet been explored in the context of oHSVs.

### Why is this important?

- The PKR evasion viruses (C134 and C154) have a marked advantage over 1<sup>st</sup> generation oHSV based upon their ability to evade the host tumor cell PKR response. Based on these findings, use of these viruses appears based on data available at this time, to represent a superior choice for examination in early Phase clinical trials.
- Utilization of entry molecule and spread in tumor. Based upon this data, nectin-1 expression may be used as a criterion for oHSV efficacy (should second-generation vectors or vectors expressing  $\gamma$ 134.5 (R7020) be utilized) and could impact on future clinical trials (for example, patients could be stratified prior to inclusion based upon known nectin-1 levels within their tumors, allowing an accurate analysis of impact on actual therapy).
- The finding that the capacity of a cell line to activate the STAT1 signaling cascade is statistically associated with resistance MPNST cell lines is a first of its kind discovery

for oHSV. This is important because future studies may show that activation of STAT1 is mediates this resistance and may be modulated with pharmaceutical inhibitors of STAT1 activation. Both ruxolitinib (a JAK1/2 inhibitor) and fludarabine (a general STAT1 inhibitor) are FDA approved drugs which can be tested as an adjuvant therapy to  $\Delta\gamma$ 134.5 oHSVs. This finding may also be important for stratifying patients by category and expected response, e.g., low/no activation of STAT1; activation of STAT1 but able to be inhibited by pharmacologic means; or activation of STAT1 and not able to be inhibited. This final group, if it exists, might be expected to be resistant to current oHSV therapies.

- Interferon stimulated genes (ISGs), the downstream targets of STAT1, are elevated at baseline in resistant cell lines prior to infection with oHSV. This may both a potential target for neo-adjuvant pharmacologic modulation (if these ISGs are stimulated by basal STAT1 activity) and may serve as biomarkers which stratify patients into predictable oHSV responsive and non-responsive categories.
- In general, MPNST tumors appear sensitive to our GLP oHSV therapeutic viruses and thus hold promise as candidate therapies for study in clinical trials in the foreseeable future.

## Reportable Outcomes

- a. Abstracts (see appendix)
- b. Manuscript in print (see appendix)
- c. Funding Research or research opportunities: None for 2014 but preparing grants for 2015

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## Appendix i

Poster session: The same poster was presented at both of the listed presentations.

UAB GBS/JHS Student Retreat, May 9 2014, “**Effect of Oncolytic Herpes Simplex Virus Replication in MPNST Cell Lines Over-Expressing Nectin-1**” Joshua D. Jackson, Adrienne McMorris, Jennifer Coleman, Justin Roth, Steven Carroll, Kevin Cassady, James Markert

Comprehensive Cancer Center Retreat, Nov. 5 2013, “**Effect of Oncolytic Herpes Simplex Virus Replication in MPNST Cell Lines Over-Expressing Nectin-1**” Joshua D. Jackson, Adrienne McMorris, Jennifer Coleman, Justin Roth, Steven Carroll, Kevin Cassady, James Markert

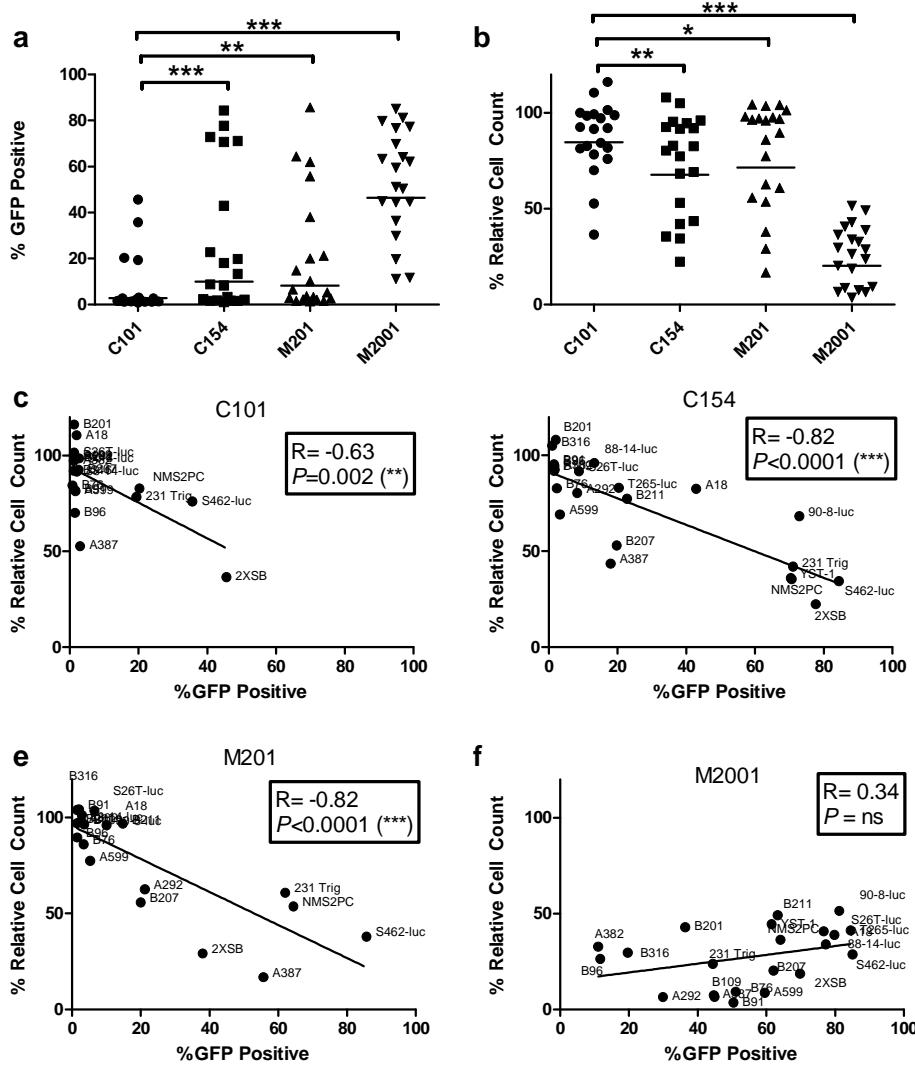
### ABSTRACT

We propose in this research to study the capacity of various oncolytic Herpes Simplex Viruses (oHSVs) to elicit regression of malignant peripheral nerve sheath tumors (MPNSTs) through virally induced cell lysis and immune recruitment. As rare and aggressive tumors of glial origin, MPNSTs frequently arise from patients with type-1 neurofibromatosis, but also form spontaneously. Five year survival ranges from 16-52%, and a lack of dependable treatment options suggests oHSV as a novel candidate to treat these malignancies. HSV is an attractive therapy because it is neurotropic and readily tolerates therapeutic transgene inserts up to 30 kb for high-level expression in infected cells. HSV has been proposed as an oncolytic therapy for tumors derived of neuronal lineage and has already been safely used in Phase I clinical trials for patients with glioblastoma multiforme. Despite promising results in select patients, others experienced less dramatic clinical response to the therapy. One potential explanation to the variability in oHSV efficacy is the absence or limiting concentration of the primary HSV entry receptor Nectin-1 on the surface of target cells. While this is a theoretical concern, it is expected that Nectin-1 concentrations in MPNST cell lines are sufficient to allow efficient viral entry. Preliminary results regarding the impact of human Nectin-1 over-expression (by lentiviral transduction in MPNST cell lines) on oHSV viral recovery are presented here. In the future, it is expected that the study of events following viral entry will explain the observed variations of in vitro data and clinical response to oHSV. Proposals involving innate viral defense responses and intracellular metabolic effects on host susceptibility, as well as correlative in vivo studies using xenogeneic and syngeneic murine models, are under development.

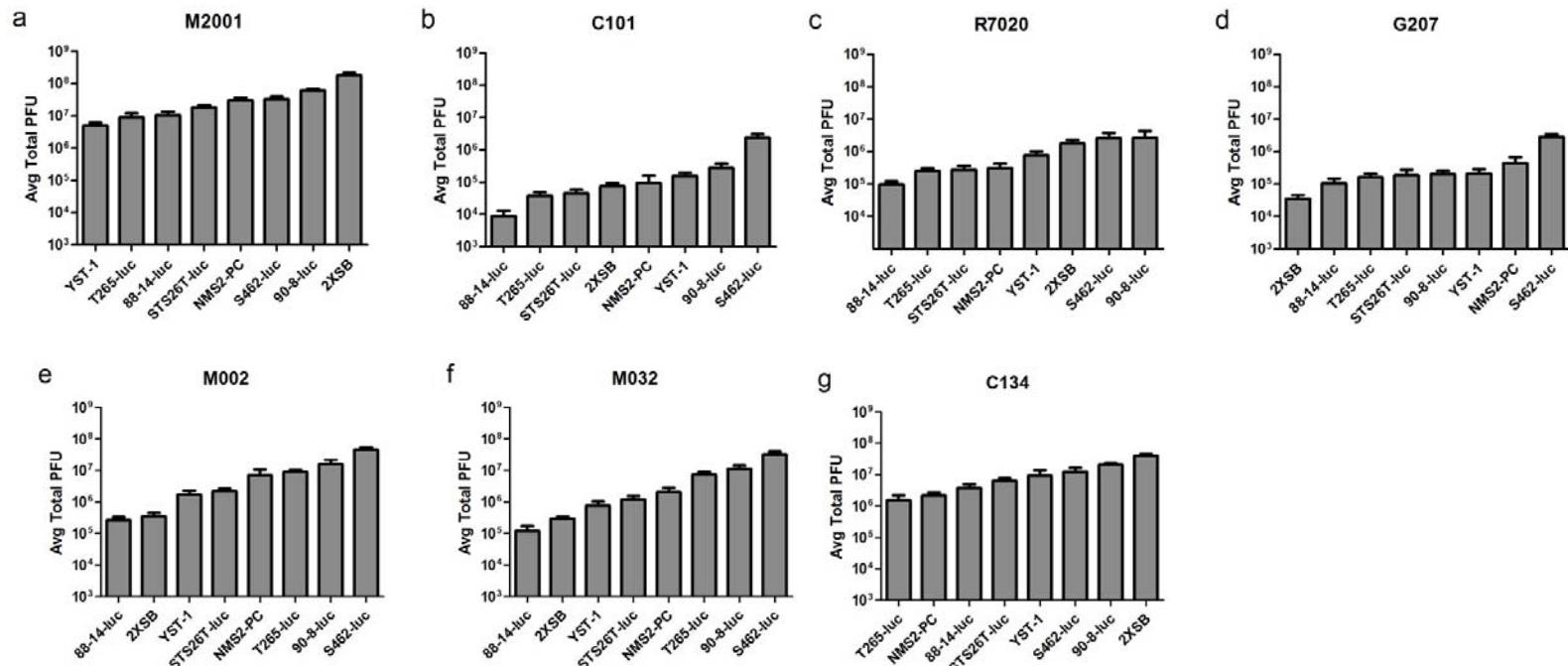
## **Appendix ii**

### **Supporting Data**

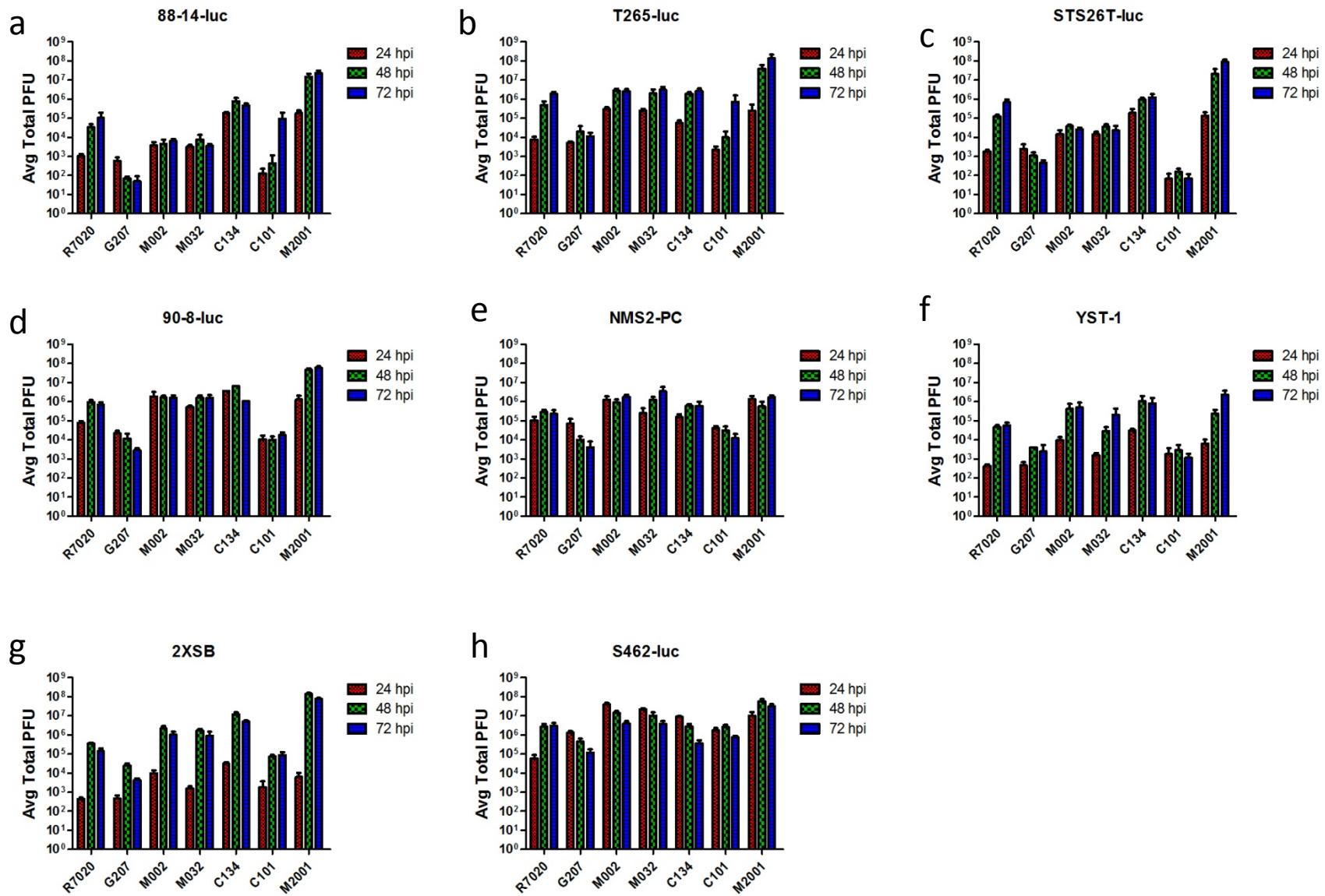
Supporting data figures can be found on the following pages.



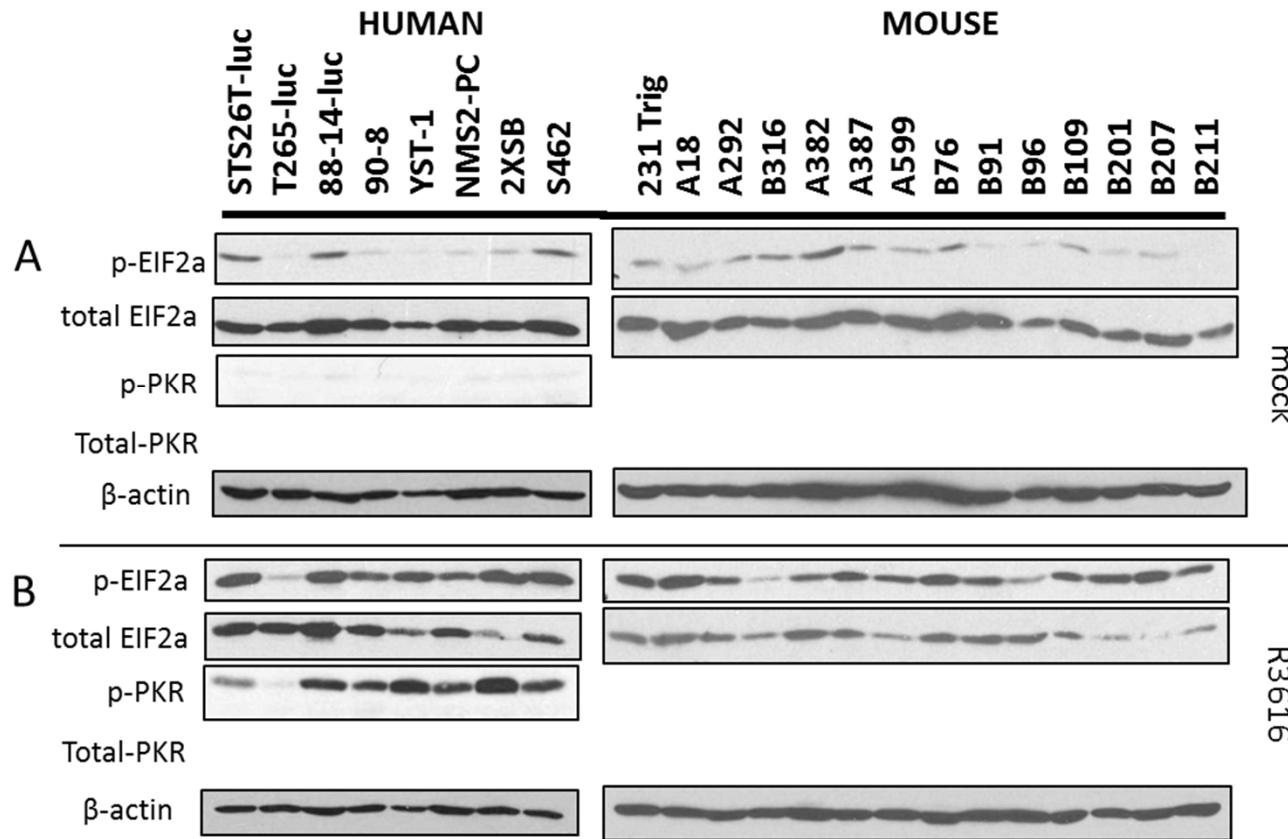
**Figure 1 (SubTask 1b): Assessment of multi-step viral productivity by GFP and cell count measurements.** All eight human and fourteen mouse MPNST cell lines were infected with the viruses C101 (R3616 + eGFP), C154 (C134 + eGFP), M201 (M002 + eGFP), or M2001 (HSV-1 F strain + eGFP) at a multiplicity of infection (MOI) of 0.1. Cells were collected at 48 hours post infection (hpi) and subjected to FACS analysis for viral GFP expression. Total cell counts for mock and infected cells were measured using standard fluorescent counting beads (Bangs Labs) according to the manufacturer's directions. Both second generation viruses C154 and M201 demonstrated significantly higher GFP positive cells (a) and reduction in relative cell counts (b) as compared to C101. As expected the wild-type virus also demonstrated these increases (a-b). Means for each group are plotted. Students paired t test was used in the statistical analysis. Data for each cell line was gathered in triplicate and averaged. To test the validity of measuring these two variables, we correlated the % GFP positive measurement and the % relative cell count for each cell line. For the attenuated viruses there was a strong and significant association with the GFP and cell count measurements (c-e) but not for the wild-type virus (f) as calculated by Pearson's correlation. Star notation defined as follows: (\*)  $P < 0.05$ , (\*\*)  $P < 0.01$ , (\*\*\*)  $P < 0.001$ .



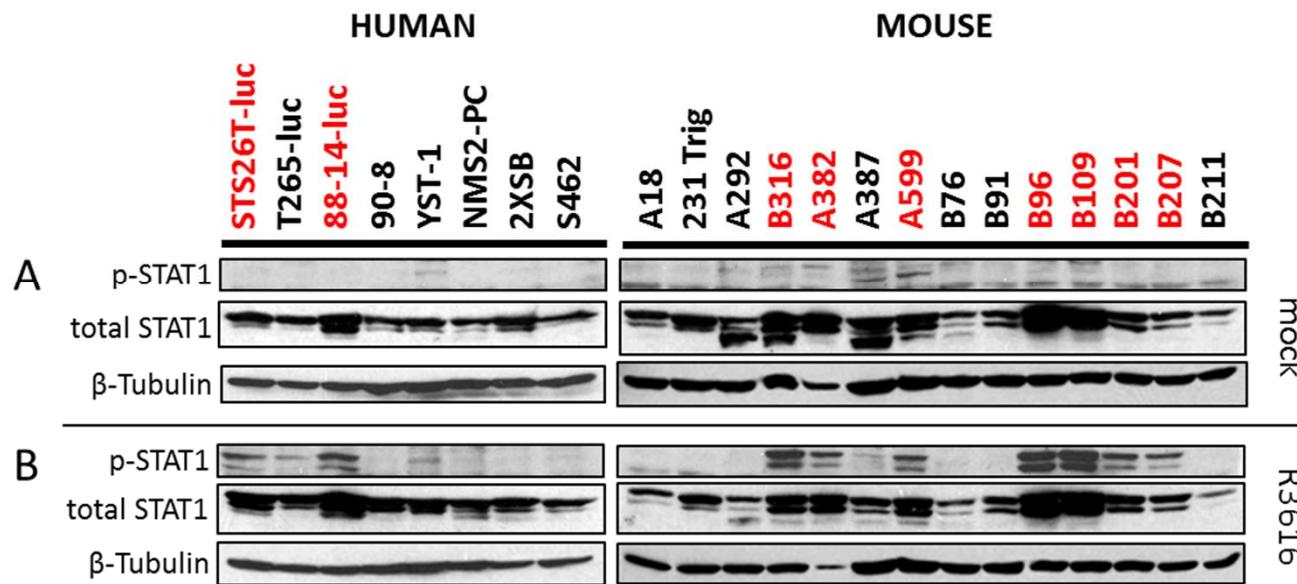
**Figure 2 (SubTask 1c): Single-step viral recovery in MPNST cell lines.** MPNST cell lines were subjected to single-step (MOI=10, 24 hr) infection by viruses M2001 (a), R7020 (b), C101 (c), G207 (d), M002 (e), M032 (f), C134 (g) and the viral titers reported as the average total plaque forming units (PFU) as determined through limiting dilution plaque formation. Error bars represent standard deviation. For each virus, a range of replication was observed between all cell lines with the largest differences observed in the double  $\gamma_134.5$  deleted viruses C101 and G207. Resistant cell lines STS26T-luc and T265-luc as well as permissive lines S462-luc and NMS2-PC were identified and selected for further study.



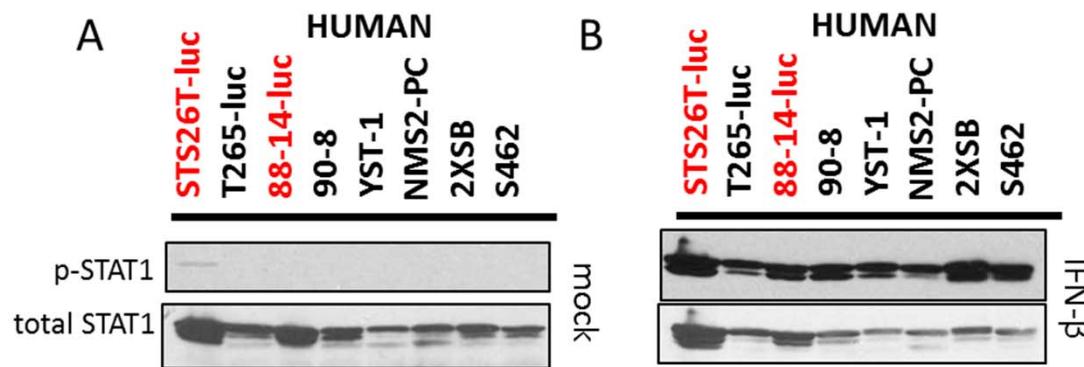
**Figure 3 (SubTask 1c): Multistep-step viral recovery in MPNST cell lines.** MPNST cell lines were subjected to multi-step (MOI=0.1 at 24, 48, and 72 hpi) infection by viruses M2001 (a), R7020 (b), C101 (c), G207 (d), M002 (e), M032 (f), C134 (g) and the viral titers reported as the average total plaque forming units (PFU) as determined through limiting dilution plaque formation. Error bars represent standard deviation. In general, permissive cell lines permit replication of attenuated viruses (e.g. C101, G207) closer to that reported for wild type M2001. Resistant lines T265-luc (b) and STS26T-luc(c) generally have suppressed replication of G207 and C101 compared to M2001.



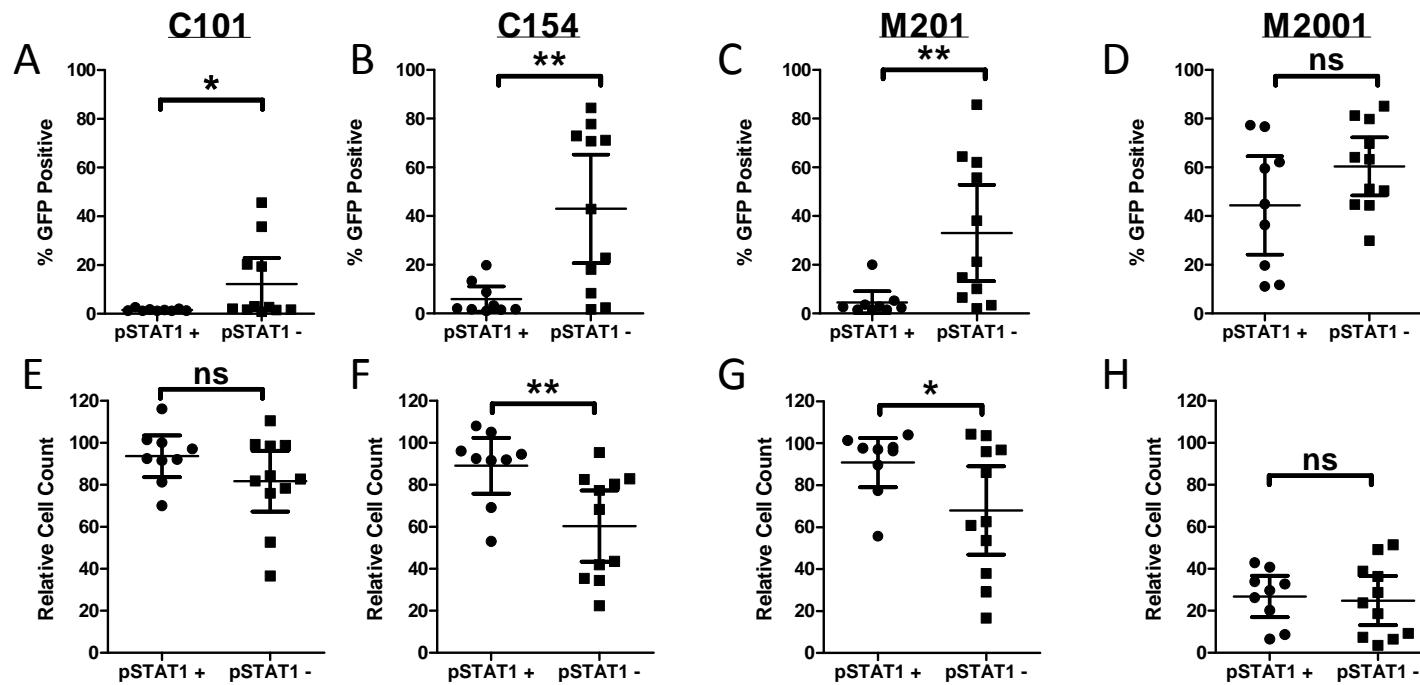
**Figure 4 (SubTask 2a): MPNST cell lines mock or R3616 infected (MOI=1, 12 hpi).** Mouse and human MPNST cell lines were mock infected (A) or infected with R3616 (MOI=1) (B) for 1 hr under normal serum (7%) conditions. Media was replaced at 1 hr. Lysates were collected in Laemmeli buffer at 12 hpi, subjected to SDS-PAGE. The following antibodies were used pEIF2a (Cell Signaling #3398), total EIF2a (Cell Signaling #5324), pPKR (Cell Signaling #3076), total PKR (Santa Cruz #SC-707), and beta actin (Sigma-Aldrich #A3853). Nearly all MPNST cell lines respond to R3616 with eIF2a phosphorylation. Human cell lines additionally demonstrate PKR phosphorylation in nearly all cell lines including all oHSV permissive cell lines.



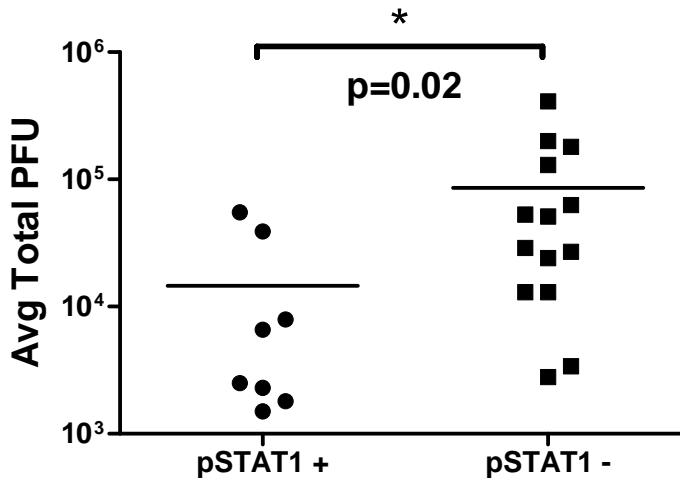
**Figure 5 (SubTask 2a): MPNST cell lines mock or R3616 infected (MOI=1, 6 hpi).** Mouse and human MPNST cell lines were mock infected (A) or infected with R3616 (MOI=1) (B) for 1 hr under normal serum (7%) conditions. Media was replaced at 1 hr. Lysates were collected in Laemmeli buffer at 6 hpi, subjected to SDS-PAGE. The following antibodies were used pSTAT1 (Y701) (Cell Signaling #9167), STAT1 (Cell Signaling #9172), and beta tubulin (Cell Signaling #2128). Only certain MPNST cell lines (red lettering) respond to R3616 with STAT1 phosphorylation.



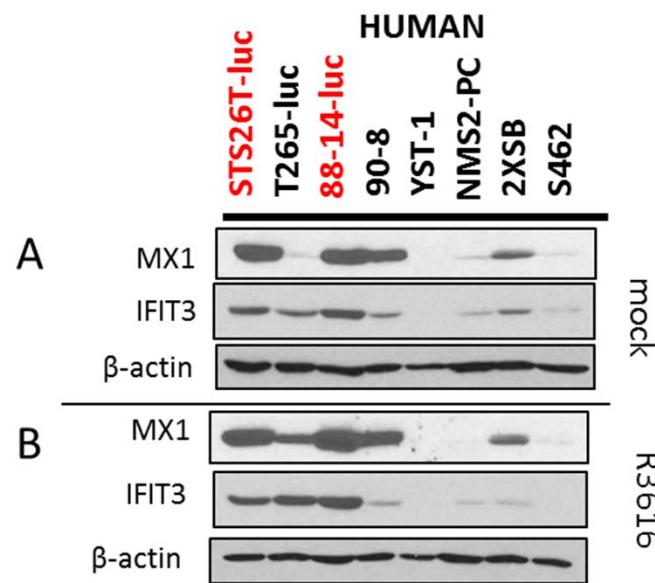
**Figure 6 (SubTask 2a): MPNST cell lines treated with or without IFN- $\beta$ .** Human MPNST cell lines were treated with fresh growth media (A) or 200 IU/ml of recombinant human IFN- $\beta$  for 30 minutes. Lysates were collected in Laemmelli buffer at 30 minutes after treatment and subjected to SDS-PAGE. The following antibodies were used pSTAT1 (Y701) (Cell Signaling #9167), STAT1 (Cell Signaling #9172). All human MPNST cell lines respond to IFN- $\beta$  by phosphorylating STAT1 including those cell lines which did not respond to R3616 with STAT1 phosphorylation (black lettering) (Figure 5).



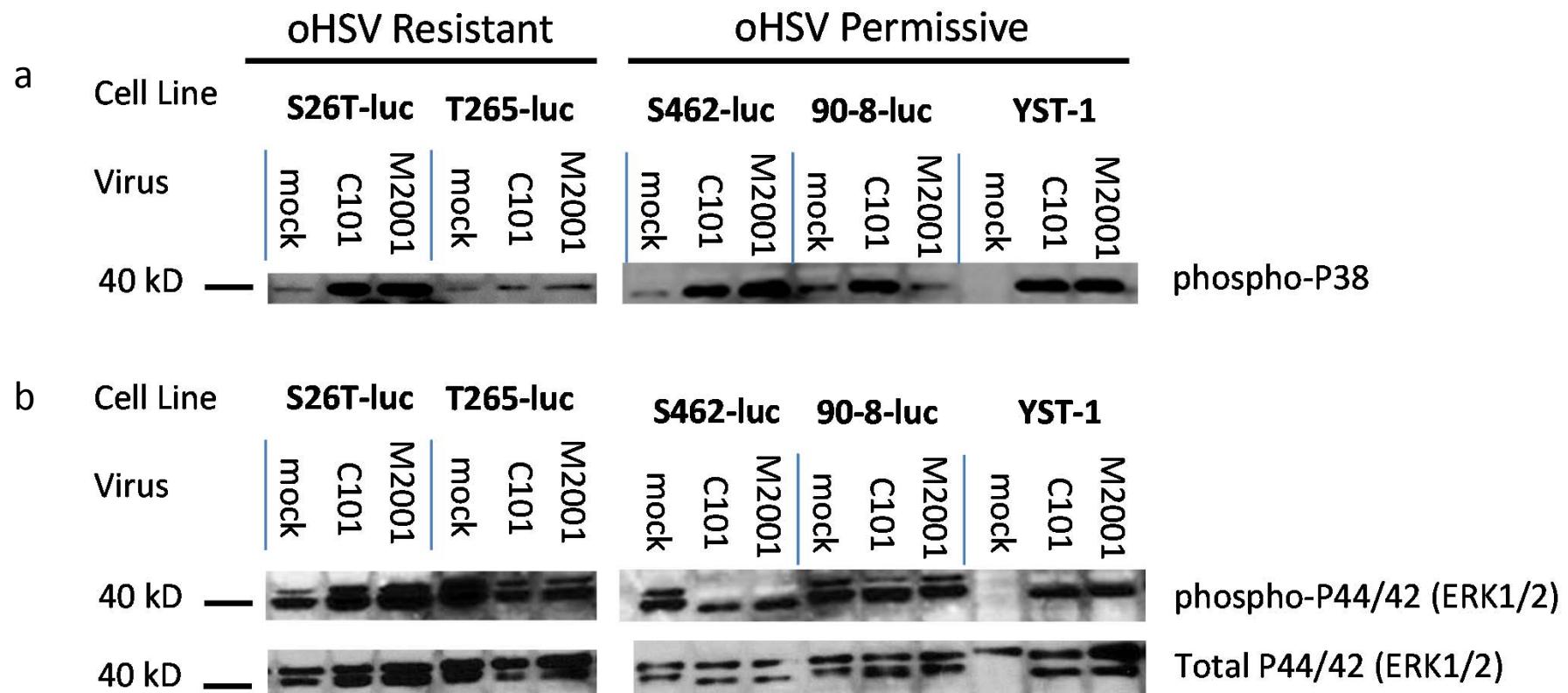
**Figure 7 (SubTask 2a): Comparison of measures of viral productivity in STAT1 responsive and unresponsive MPNST cell lines.** Data obtained from Figure 1 was grouped into STAT1 responsive and unresponsive groups based upon the observations made in Figure 5. Student's t test with Welch's correction were performed for each group. There was significant association with the STAT1 response for the oHSVs C101, C154, and M201 by both measurements of %GFP positive (A-C) and % relative cell count (E-G) except for the % relative cell count in C101 (E). No significance was observed for either measurement for the wild-type virus M2001 (D and H) indicating that the ability of the cell to activate STAT1 is irrelevant during infection with the wild-type HSV-1. Star notation defined as follows: (\*)  $P<0.05$ , (\*\*)  $P<0.01$ , (\*\*\*)  $P<0.001$ .



**Figure 8 (SubTask 2a): Comparison of measures of R3616 replication capacity in STAT1 responsive and unresponsive MPNST cell lines.** All human and mouse MPNST cell lines were infected with the  $\Delta\gamma_134.5$  oHSV R3616 at an MOI of 1 for 24 hpi. Cells were titered in the standard fashion (Jackson *et al.*) and viral plaques counted in triplicate and reported as the average total plaque forming unit (PFU). Grouping of cell lines by STAT1 response as determined in Figure 5 revealed statistically significant lower titers of R3616 in cells which could respond to the virus by activation of the STAT1 pathway.



**Figure 9 (SubTask 2a): ISG expression by human MPNST cell lines mock or R3616 infected (MOI=1, 12 hpi).** Human MPNST cell lines were mock infected (A) or infected with R3616 (MOI=1) (B) for 1 hr under normal serum (7%) conditions. Media was replaced at 1 hr. Lysates were collected in Laemmeli buffer at 12 hpi, subjected to SDS-PAGE. The following antibodies were used: MX1 (ProteinTech #13750-1-AP), IFIT3 (ProteinTech # 15201-1-AP), and beta actin (Sigma-Aldrich #A3853). Cell lines which were capable are STAT1 responsive (red lettering) show high basal levels of ISGs prior to infection whereas permissive lines S462, NMS2-PC, and YST-1 do not.



**Figure 10 (Subtask 2b and d): P38 and ERK1/2 activation in resistant and permissive cell lines.** MPNST cells were infected with mock, C101 or M2001 virus at an MOI of 10 and lysates collected 12 hpi. Immunoblots for phospho-P38 (a) indicate that P38 is activated in all permissive cell lines upon infection as well as the resistant line S26T-luc. ERK 1/2 activation is apparent in all resistant and permissive MPNST cell lines (b). Basal levels of ERK 1/2 activation are apparent in all mock infected MPNST cell lysates except the permissive line YST-1 (b).

### **Appendix iii**

Manuscript published in *Gene Therapy* can be found on the following pages.

## SHORT COMMUNICATION

# Assessment of oncolytic HSV efficacy following increased entry-receptor expression in malignant peripheral nerve sheath tumor cell lines

JD Jackson<sup>1</sup>, AM McMorris<sup>1</sup>, JC Roth<sup>2</sup>, JM Coleman<sup>1</sup>, RJ Whitley<sup>2</sup>, GY Gillespie<sup>1,3</sup>, SL Carroll<sup>4,5</sup>, JM Markert<sup>1,2,6</sup> and KA Cassady<sup>2,6</sup>

Limited expression and distribution of nectin-1, the major herpes simplex virus (HSV) type-1 entry-receptor, within tumors has been proposed as an impediment to oncolytic HSV (oHSV) therapy. To determine whether resistance to oHSVs in malignant peripheral nerve sheath tumors (MPNSTs) was explained by this hypothesis, nectin-1 expression and oHSV viral yields were assessed in a panel of MPNST cell lines using  $\gamma$ 134.5-attenuated ( $\Delta\gamma$ 134.5) oHSVs and a  $\gamma$ 134.5 wild-type (wt) virus for comparison. Although there was a correlation between nectin-1 levels and viral yields with the wt virus ( $R = 0.75$ ,  $P = 0.03$ ), there was no correlation for  $\Delta\gamma$ 134.5 viruses (G207, R7020 or C101) and a modest trend for the second-generation oHSV C134 ( $R = 0.62$ ,  $P = 0.10$ ). Nectin-1 overexpression in resistant MPNST cell lines did not improve  $\Delta\gamma$ 134.5 oHSV output. While multistep replication assays showed that nectin-1 overexpression improved  $\Delta\gamma$ 134.5 oHSV cell-to-cell spread, it did not confer a sensitive phenotype to resistant cells. Finally, oHSV yields were not improved with increased nectin-1 *in vivo*. We conclude that nectin-1 expression is not the primary obstacle of productive infection for  $\Delta\gamma$ 134.5 oHSVs in MPNST cell lines. In contrast, viruses that are competent in their ability to counter the antiviral response may derive benefit with higher nectin-1 expression.

Gene Therapy advance online publication, 14 August 2014; doi:10.1038/gt.2014.72

## INTRODUCTION

Malignant peripheral nerve sheath tumors (MPNSTs) are a highly aggressive cancer of the peripheral nervous tissue believed to originate within the Schwann cell lineage<sup>1</sup> and are most commonly associated with the genetic condition neurofibromatosis type-1. Treatment options for MPNSTs beyond surgery are inadequate, resulting in a median survival of only 26 months.<sup>2</sup> Oncolytic virotherapy by attenuated herpes simplex type-1 viruses (oHSVs) has been proposed as an alternative to chemotherapy and radiotherapy for the treatment of MPNSTs.<sup>3–7</sup> HSVs with  $\gamma$ 134.5 neurovirulence gene deletions are safe in humans and have been shown to selectively replicate in tumor cells.<sup>8</sup> These attenuated  $\Delta\gamma$ 134.5 oHSVs have a clinically verified safety profile in patients with malignant glioma and have been associated with measurable antitumor responses.<sup>9–13</sup> However, these patient responses have varied widely, likely due to tumor susceptibility. Therefore, we have sought to further elucidate the mechanisms of oHSV resistance.

In our initial investigation into potential oHSV resistance mechanisms within MPNSTs, we have tested the hypothesis that oHSV resistance is attributable to the insufficient expression of HSV-1 entry receptors by tumor cells.<sup>14–18</sup> Four viral glycoproteins (gD, gB and gH/gL) and a cellular glycoprotein D (gD)-interacting receptor have been demonstrated as necessary and sufficient to trigger cellular entry.<sup>19–23</sup> Of the three cellular HSV-1 gD-interacting

receptors, nectin-1, a cellular adhesion protein expressed in epithelial cells,<sup>24</sup> fibroblasts and neurons,<sup>25</sup> has been proposed as the major HSV-1 entry receptor.<sup>26</sup> Herpes virus entry mediator (HVEM)<sup>27</sup> and 3-O-sulfated heparan sulfate (3-OS-HS)<sup>28</sup> have also been demonstrated to facilitate HSV-1 entry. Additional cell-surface molecules that interact with other viral glycoproteins have been identified, though the broad necessity of these in permitting HSV-1 infection and spread remains to be determined and the lack of these molecules has not yet been implicated in limiting the oncolytic capacity of oHSV.

Here, we have investigated the hypothesis that HSV entry-receptor expression is a determinant of oHSV efficacy in MPNST cells and have identified whether an increase in entry-receptor expression improves the viral yield and spread of oHSVs. The influence of entry-receptor expression was examined in the context of an array of viral genotypes, including a representative wild-type (wt)  $\gamma$ 134.5 HSV-1, a fully attenuated  $\Delta\gamma$ 134.5 oHSV, and an attenuated second-generation oHSV capable of host antiviral evasion. We report the following conclusions: (1) correlation of nectin-1 expression with viral production capacity appears more important in viruses which are genetically competent to counter the intrinsic antiviral response, (2) increased expression of entry-receptor molecules modestly improves cell-cell spread of  $\Delta\gamma$ 134.5 oHSVs, but yields little benefit to viral production and (3) increases in entry-receptor expression do not render resistant MPNST cell lines permissive to  $\Delta\gamma$ 134.5 oHSV infection.

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## RESULTS AND DISCUSSION

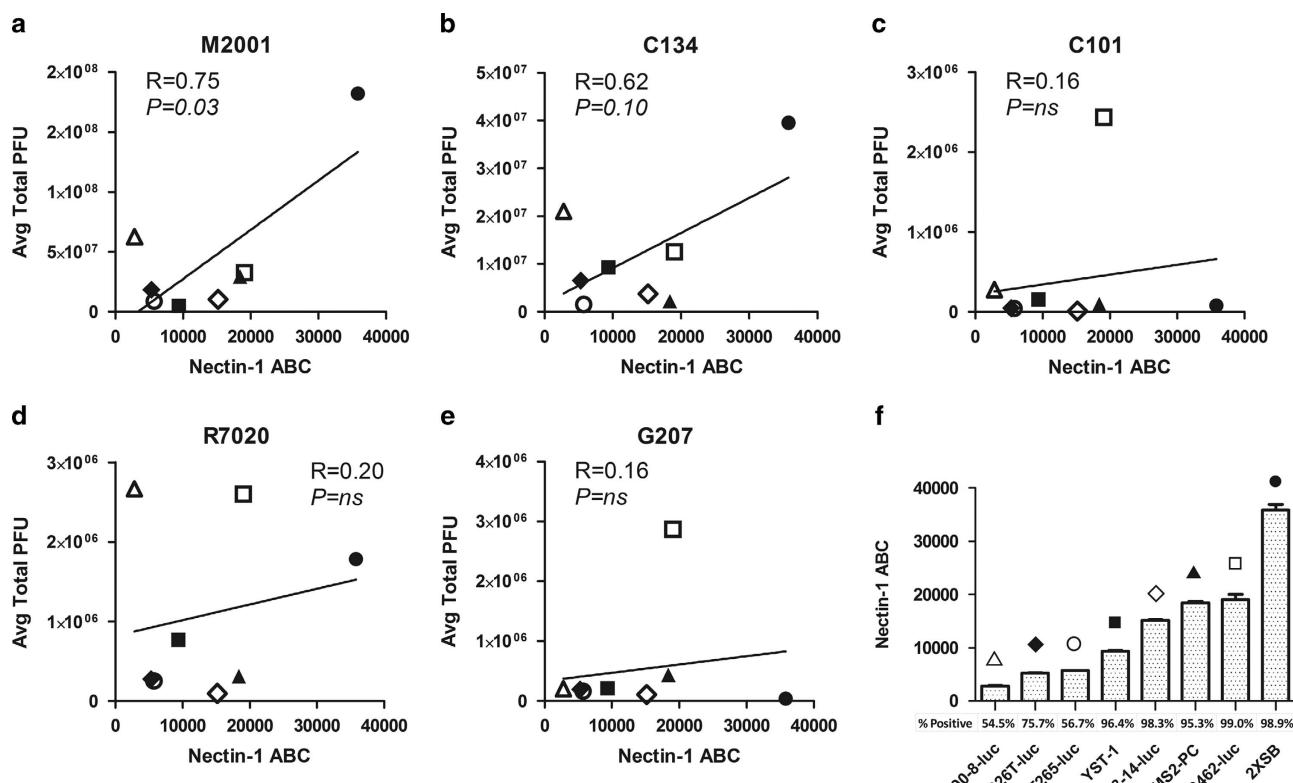
MPNST cell lines have been previously identified as susceptible to oHSV infection and cytotoxicity.<sup>3</sup> To examine the correlation between viral production capacity and entry-receptor expression, human MPNST cell lines STS-26T, T265-2c, NMS2-PC, S462, YST-1, 90-8, ST88-14 and 2XSB, or their luciferase-expressing derivatives ('-luc'), were first infected at a multiplicity of infection (MOI) of 10 (single-step replication assay) with a panel of genetically modified HSV-1 and cellular lysates collected 24 h post infection (h.p.i.) for viral recovery analysis (Supplementary Figure 1). The viral yields from this assay are presented in correlation with nectin-1 expression in Figures 1a–e.

HSV-1 entry receptors have not been previously identified in MPNSTs or cells of the Schwann cell lineage. Upon examination of nectin-1 and HVEM expression in our panel of MPNST cell lines, we found detectable levels of nectin-1 in all of the lines, with population-wide (>95%) expression of nectin-1 in five of eight cell lines (Figure 1f). Population-wide expression of HVEM in MPNST cell lines was observed in only one of eight lines (Supplementary Figure 2D); therefore, HVEM was excluded as a candidate for the major entry receptor in MPNSTs. The other established entry receptor 3-OS-HS was not examined in this study due to the lack of a commercially available antibody. However, HSV-1 infection of the resistant cell lines selected for further study (STS26T-luc and T265-luc) was found to be dependent on nectin-1 expression alone by nectin-1 neutralization assays (Supplementary Figure 2).

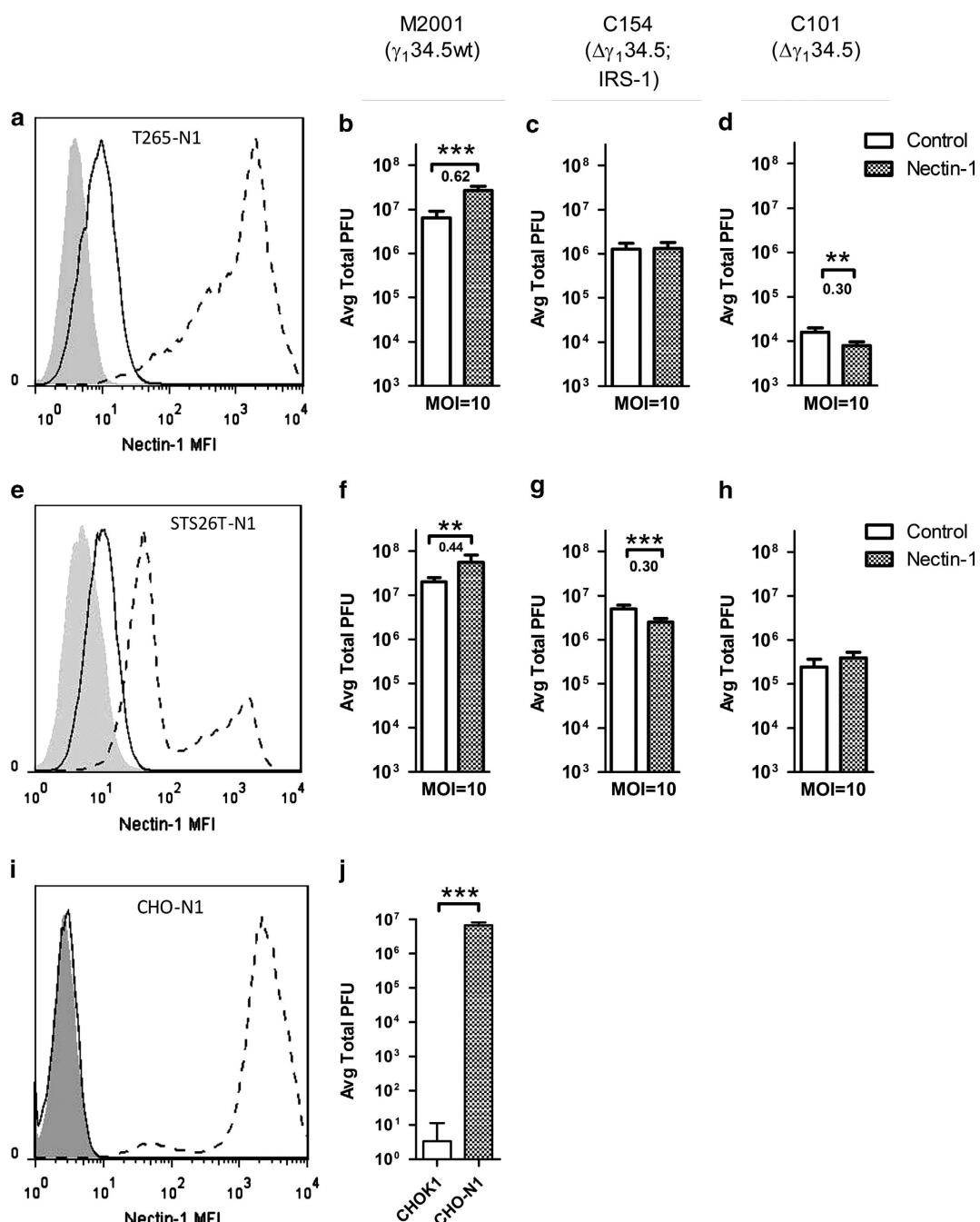
Pearson's correlation coefficients were calculated between viral recovery data and nectin-1 expression levels. Possible positive associations were found for the wt M2001 ( $R=0.75$ ;  $P=0.03$ ) and attenuated second-generation oHSV C134 ( $R=0.62$ ,  $P=0.10$ ) viruses (Figures 1a and b). While prior studies involving thyroid cancer<sup>14</sup> and head and neck squamous cell carcinoma<sup>15</sup> cell lines demonstrated correlation between viral yields of oHSV NV1023 (a derivative of R7020) and nectin-1 expression, no such associations were observed for G207, C101 or R7020 oHSVs suggesting that the specific viral genotype influences the outcome of infection.

The lack of a clear association of nectin-1 expression with attenuated  $\Delta\gamma$ 34.5 oHSV viral yields led us to further evaluate the functional impact of increased entry-receptor expression on oHSV sensitivity. To assess this, the oHSV-resistant cell lines, T265-luc and STS26T-luc were transduced with full-length human nectin-1 (nectin-1a) using lentivirus LV2114CK. An mCherry expressing lentivirus was used as a control and confirmed that transduction alone did not alter viral production (data not shown).

If low entry-receptor expression diminishes the ability of oHSV to establish an initial infection, we would predict that increased nectin-1 expression would increase the initial opportunity for entry, resulting in replication within a greater number of cells and an increase in the total production of virus. To determine the impact of increased nectin-1 expression on viral yields in MPNSTs, single-step (MOI=10, 24 h.p.i.) and multistep (MOI=0.1, 24, 48, 72 h.p.i.; Supplementary Figure 3) viral recovery assays were performed using the parent and nectin-1 transduced cell lines. Because the



**Figure 1.** Correlation of nectin-1 expression with viral titers. Pearson's correlation coefficients (a–e) were calculated between the viral titration data from M2001, C134, C101, R7020, G207 (Supplementary Figure 1) and the nectin-1 expression levels (f) from cell lines 90-8-luc (open triangle), STS26T-luc (closed diamond), T265-luc (open circle), YST-1 (closed square), 88-14-luc (open diamond), NMS2-PC (closed triangle), S462-luc (open square), and 2XSB (closed circle). A strong and significant correlation was noted for M2001. Cells were infected in triplicate by a single-step replication assay (MOI=10) and the lysates collected and titrated at 24 h.p.i. Nectin-1 expression was quantified by flow cytometry after incubation with phycoerythrin (PE)-conjugated mouse monoclonal antibody with subsequent quantification using antibody quantification beads. The percentage of the cell population staining above the isotype control is also reported. Receptor quantification was performed in triplicate with the standard deviation reported.



**Figure 2.** Overexpression of nectin-1 in resistant cells and impact on single-step replication assays. Nectin-1 was transduced via lentivirus into oHSV-resistant cell lines T265-luc (a) and STS26T-luc (e) as well as control cell line CHO-K1. Isotype control (shaded), parent (solid line) and transduced (dashed line) cell lines are shown. Transduction of the nectin-1-deficient cell line (i) demonstrated function as an entry receptor as apparent by M2001 replication (j). The impact of nectin-1 overexpression in resistant cell lines was tested by single-step (MOI = 10) replication by viruses M2001 (b, f), C154 (c, g) and C101 (d, h) and compared with control cell lines. Significance was determined by two-tailed Student's *t*-test with unequal variance. Significance was set at  $P < 0.05$ . For cells with significant changes in titer, the logarithm of the absolute value of the increase was reported below the significance marking. Changes in titer greater than 0.5 log are considered to be biologically relevant. \*\* $P > 0.01$  and \*\*\* $P > 0.001$ .

reliable titration repeatability of HSV is within approximately 0.5 log, only changes in titer greater than 0.5 log are considered to be biologically relevant. The nectin-1 transduction of T265-luc and STS26T-luc resulted in abundant nectin-1 expression in T265-N1 and STS26T-N1 cell lines, respectively (Figures 2a and e). While increased entry-receptor expression improved the yields of a representative wt virus (Figures 2b and f), the increased expression did not improve the titers of a next-generation oHSV C154, an EGFP

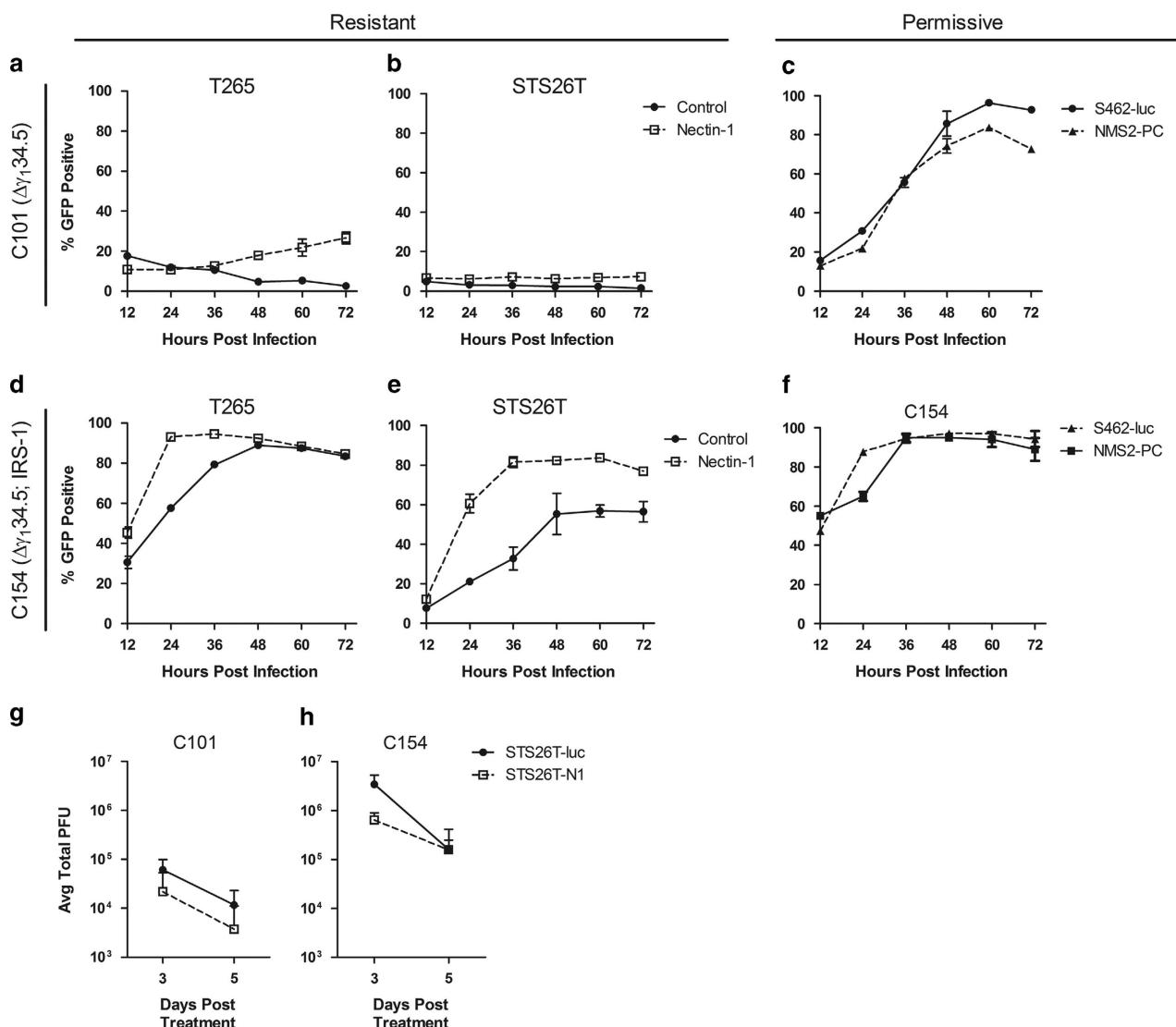
expressing variant of C134 (Figures 2c and g) or first-generation  $\Delta\gamma$ 34.5 oHSV C101 (Figures 2d and h). To demonstrate that increased nectin-1 expression would be expected to improve viral production, the HSV receptor-deficient cell line CHO-K1 was transduced with nectin-1. A significant and greater than 5 log increase in wt HSV-1 titers was observed (Figures 2i and j).

Interaction with HSV entry receptors is essential for initial HSV entry as well as the subsequent cell-to-cell spread of HSV.<sup>29</sup>

To assess the effect of increased nectin-1 expression on viral spread, we measured viral GFP expression in MPNST cells over the time in multistep assays following infection with GFP expressing C101 and C154 (Figure 3) or M2001 (Supplementary Figure 4). The results show that nectin-1 overexpression improved the ability of C101 to undergo cell-to-cell spread and increased the proportion of cells infected from 3 to 27% and from 1 to 7% of the cell population in T265-N1 and STS26T-N1 in multistep replication assays (MOI = 0.1, 24, 48 and 72 h.p.i.) respectively (Figures 3a and b). Despite this improved spread in resistant lines, the maximum spread was much less than that observed in the naturally permissive S462-luc and NMS-2PC MPNST cell lines, where C101 was capable of infecting >80% of the cells (Figure 3c). This suggests that endogenous levels of entry receptors are sufficient to permit infection and sustain  $\Delta\gamma_{134.5}$  oHSV spread in these lines and that increased entry-receptor expression is not sufficient to render resistant cell lines with a permissive phenotype. Of note, the overexpressed nectin-1 levels far exceeded the highest

endogenous levels in the permissive lines (Supplementary Figure 5), suggesting that restricted entry is not an explanation for MPNST resistance to oHSVs. This conclusion is further supported by the fact that infection of the same cell lines with a second-generation oHSV (that is, C134 or C154) capable of evading the antiviral response<sup>30</sup> resulted in approximately 10–100 fold increase in viral titers and notably greater cell-to-cell spread as compared with C101 (Figures 3d and e).

To determine the extent to which *in vivo* studies recapitulated these results, athymic nude mice were engrafted with either parent or nectin-1 expressing cell lines. Of the resistant cell lines, only STS26T-luc and the nectin-1 overexpressing variant established flank tumors. Tumors were injected with  $1 \times 10^7$  plaque forming units of C101 or C154, and viral recovery was measured on days 3 and 5 post injection. Similar to the *in vitro* results (Figure 3g and h), the next-generation virus had a >10-fold viral production advantage over the  $\Delta\gamma_{134.5}$  oHSV C101, however neither virus demonstrated an increased viral titer between days 3



**Figure 3.** Impact of increased nectin-1 expression on oHSV spread *in vitro* and viral recovery *in vivo*. Resistant cell lines STS26T-luc and T265-luc and their nectin-1 transduced variants, as well as permissive cell lines S462-luc and NMS2-PC, were infected in a multistep assay (MOI = 0.1) with fully attenuated oHSV C101 (a–c) or second-generation C154 expressing HCMV IRS1 (d–f) and monitored by flow cytometry over time for viral infection as evident by expression of viral GFP. STS26T-luc and STS26T-N1 cells were engrafted in the flanks of nude mice and following tumor formation were injected with  $1 \times 10^7$  plaque forming units (PFU) of C101 or C154. Tumors were harvested and viral titers determined at days 3 and 5 following infection (g and h). Data are representative of four tumors with standard deviation reported.

and 5 even with increased nectin-1 expression. Tumors were also collected for immunohistochemistry and staining for HSV-1 confirmed that increased nectin-1 expression did not benefit oHSV spread between days 3 and 5 (data not shown). The *in vivo* results therefore confirmed that neither the first- nor the second-generation oHSVs derived a benefit to viral output from increased entry-receptor expression.

In summary, the work presented here provides insight into one of the proposed determinants of oHSV therapeutic efficacy. We conclude that the primary mode of MPNST resistance to  $\Delta\gamma$ 34.5 oHSVs is not due to limited expression of nectin-1. Despite the primary conclusions of previously published work that entry-receptor expression is predictive of a productive infection by oHSV, we suggest that the use of viruses in these previous studies which contained at least one functional copy of the  $\gamma$ 34.5 gene (NV1023)<sup>14,15,18</sup> or  $\gamma$ 34.5 under a nestin promoter (rQnestin34.5)<sup>16</sup> is in line with our conclusions that viruses which are genetically competent to counter the intrinsic antiviral response benefit the most from increased entry-receptor expression. Similarly in our work, the wt HSV-1 and C134 viruses derived greater benefit from higher entry-receptor expression than did the first-generation  $\Delta\gamma$ 34.5 oHSVs. Furthermore, the work of Wang *et al*<sup>16</sup> showed that only the  $\gamma$ 34.5 containing virus was able to substantially benefit from increased nectin-1 expression while the  $\Delta\gamma$ 34.5 control virus did not. Future work should therefore include the characterization of the capacity for an intrinsic antiviral response as the major mechanism for oHSV resistance in MPNSTs.

## MATERIALS AND METHODS

### Cell lines

MPNST cell lines STS26T-luc, T265-luc, ST88-14-luc, S462-luc, 90-8-luc, NMS2-PC, YST-1 and 2XSB were provided by Dr Steve Carroll (University of Alabama at Birmingham). Cell lines STS26T-luc, T265-luc, and ST88-14-luc express firefly luciferase and have been previously described.<sup>31</sup> S462-luc and 90-8-luc were transduced *via* lentivirus to express Renilla luciferase. HSV-1 entry receptor-deficient cell line CHO-K1 was generously provided by Dr Yancey Gillespie (University of Alabama, Birmingham). All MPNST cell lines were maintained in DMEM, 10% FBS, and 1% P/S. CHO-K1 cells were maintained in Ham's F12, 10% FBS and 1% P/S. Vero cells were obtained from American Type Culture Collection (Manassas, VA, USA) and maintained in MEM and 5% BGS. All cell lines were confirmed to be free of Mycoplasma by DAPI staining and PCR detection.

### Viruses

All viruses have been previously described. Briefly, M2001 was constructed by insertion of the gene encoding EGFP under the control of the CMV immediate early promoter into the U<sub>L</sub>3-U<sub>L</sub>4 intergenic region of the prototypical wt HSV-1 (F) strain.<sup>32</sup> C101 and C134 were derived from the  $\Delta\gamma$ 34.5 mutant HSV-1 R3616 by insertion respectively of the EGFP or HCMV *IRS1* genes under the control of the CMV immediate early promoter in the U<sub>L</sub>3-U<sub>L</sub>4 intergenic region.<sup>33</sup> C154 is derived from C134 by insertion of EGFP into the deletion loci of  $\gamma$ 34.5. G207 (Medigene, Inc., San Diego, CA, USA) is a clinical grade oHSV derived from R3616 with the additional insertion of *lacZ* in the U<sub>L</sub>39 region.<sup>34</sup> R7020 (kindly provided by Bernard Roizman; University of Chicago, Chicago, IL, USA), is a clinical grade oHSV derived from HSV-1 (F) strain by insertion of a region of the HSV-2 genome encoding glycoproteins G, D, I and a portion of E into one of the internal repeat regions of HSV-1 (F) disrupting one copy of the neurovirulence gene  $\gamma$ 34.5.<sup>35</sup>

### Viral titering assays

Viral titers were determined by limiting dilution plaque formation assays as previously described.<sup>33</sup> MPNST cells were incubated for 2 h with virus diluted in 100  $\mu$ l infection media (DMEM + 1% FBS) and replaced with growth media after infection. An equivalent volume of sterile milk was added and the plate subjected to three cycles of freeze-thaw at  $-80^{\circ}\text{C}$ . Lysate was collected, sonicated, serially diluted in Vero infection media (MEM + 1% BGS), and incubated on Vero monolayers. Infection media was replaced with growth media containing 0.01% human AB serum (Corning Cellgro, Corning, NY, USA). After 48 h, plaques were counted following May-Grunwald/methanol staining as previously described. All experiments were performed in triplicate and the average total plaque forming units reported with standard deviation.

### Viral entry-receptor quantification

Expression of entry receptors was quantified by flow cytometry using either phycoerythrin-conjugated mouse monoclonal antibodies to nectin-1 (R1.302) (Biolegend, San Diego, CA, USA), HVEM (Biolegend), or isotype control (BD Biosciences, San Jose, CA, USA). Antibody concentrations used were confirmed to be saturating. Cells analyzed using a FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Concurrently, Quantum Simply Cellular beads (Bangs Laboratories, Fisher, IN, USA) were used to determine the antibody binding capacity of each cell line. Mean fluorescence analysis was performed using FlowJo (v 7.6.1; Tree Star, Ashland, OR, USA) and the receptor-mean fluorescence intensity data converted to antibody binding capacity using a script provided with the Quantum Simply Cellular kit. All measurements were averaged from three independently seeded wells, and the final antibody binding capacity reported as the difference above the isotype control.

### Correlation of nectin-1 and viral recovery

Pearson's correlation coefficients between nectin-1 expression and viral recovery were determined by analysis of the data in Prism 5 (GraphPad Software, La Jolla, CA, USA). Cutoff for statistical significance was set at  $P < 0.05$ .

### Nectin-1 overexpression

A self-inactivating lentiviral vector was used to overexpress nectin-1 or control mCherry in oHSV-resistant cell lines. Human nectin-1 clone (Clone ID: 8322523) was obtained from Open Biosystems (Thermo Scientific, Waltham, MA, USA). Nectin-1 cDNA was PCR amplified using primers 5'-CGGATCCGGGTCGACCCGATGGCTC GGATGGGGCTT-3' and 5'-CCGGGTCGAGCGGCCGCGCTACACGTAC CACTCCTCTTGGAA-3' (IDT, Coralville, IA, USA) in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The recipient vector has been previously described.<sup>36</sup> An intermediate lenti-vector was first constructed by insertion of an IRES-puromycin *N*-acetyl-transferase cassette into the *NotI*- and *EcoRI*-digested pLVmnd lentivirus. The intermediate construct was then digested with *Sall* and *NotI* for subsequent insertion of the PCR-amplified Nectin-1 cDNA. The sequence of the coding region of the resulting lentiviral vector pCK2114 was verified. The control lentivirus (pLVmnd.CIP) was similarly constructed by insertion of mCherry upstream of the IRES-puromycin *N*-acetyl-transferase cassette in the intermediate virus. Lentiviruses were produced by co-transfection of pCK2114 or pLVmnd.CIP with pMD.G (VSVG pseudotype), and pCMV. deltaR8.91 (HIV packaging) in 293T cells using Lipofectamine 2000 and OptiMEM media (Gibco, Carlsbad, CA, USA). After 12 h, transfection media was replaced with DMEM/F12 with 10% FBS. Lentiviral-enriched supernatant was collected 48 h post transfection, filtered through a 0.22-micron filter, and mixed with polybrene (8  $\mu\text{g ml}^{-1}$ ). STS26T-luc and T265-luc cells were

subsequently transduced with the resulting lentiviruses enriched by puromycin selection ( $5\text{ }\mu\text{g ml}^{-1}$ ), followed by fluorescence activated cell sorting to obtain pure populations (UAB Comprehensive Flow Cytometry Core, Birmingham, AL, USA).

#### HSV titers in nectin-1 overexpressing cell lines

The impact of increased nectin-1 expression on viral titers in resistant cell lines was determined as described above with the exception of replacing infection media with MPNST growth media and 0.01% human AB serum to minimize extracellular spread of the virus. Statistical significance was determined by two-tailed Student's *T*-test assuming equal variance. Star notation indicating significant differences is as follows: (\*) for  $P < 0.05$ , (\*\*) for  $P < 0.01$  and (\*\*\*) for  $P < 0.001$ .

#### HSV spread as measured by GFP expression

Cells were incubated with virus or mock infected for 2 h at  $\text{MOI} = 0.1$  in infection media. Infection media was replaced with growth media and 0.01% human AB serum following incubation. Cells were harvested at 12 h intervals and analyzed by flow cytometry. The percent GFP-positive measurement was assessed by defining the GFP (FL1) gate at 1% positive of the mock-treated cells. The percentage of the infected cell population expressing GFP was then recorded. All data points were performed in triplicate, averaged, and the standard deviation reported.

#### In vivo viral recovery

Six-week-old athymic nude (nu/nu) mice (NCI-Frederick, Frederick, VA, USA) were obtained and allowed to adjust for a period of 2 weeks. Bilateral, subcutaneous tumors were engrafted in the flank by injection of  $5 \times 10^6$  cells suspended in 50:50 BD Matrigel (Becton Dickinson) and serum-free DMEM. Four tumors were used for each virus and timepoint. When tumors reached an average size of  $300\text{ mm}^3$ ,  $1 \times 10^7$  plaque forming units of oHSV suspended in saline-buffered solution was injected intratumorally. At days 3 and 5 following infection, mice were euthanized and tumors recovered in DMEM and kept on ice. After tumors were mechanically dissociated, an equivalent volume of sterile milk was added to the homogenate and viral titering was performed as described above. Titers are plotted with standard deviation.

#### CONFLICT OF INTEREST

JMM, GYG, and RJW are co-founders, stockholders and consultants for Catherex, Inc., which holds intellectual property related to oncolytic HSV.

#### ACKNOWLEDGEMENTS

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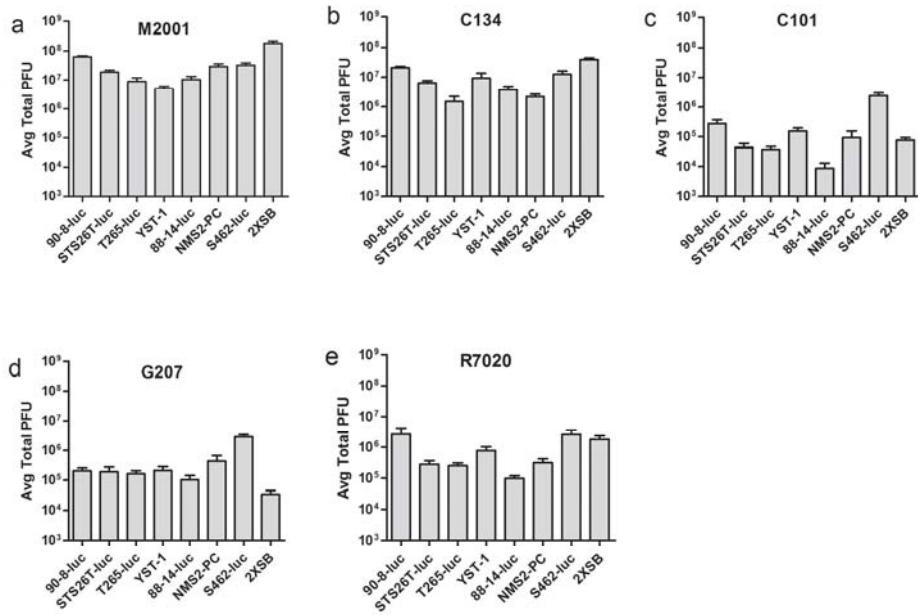
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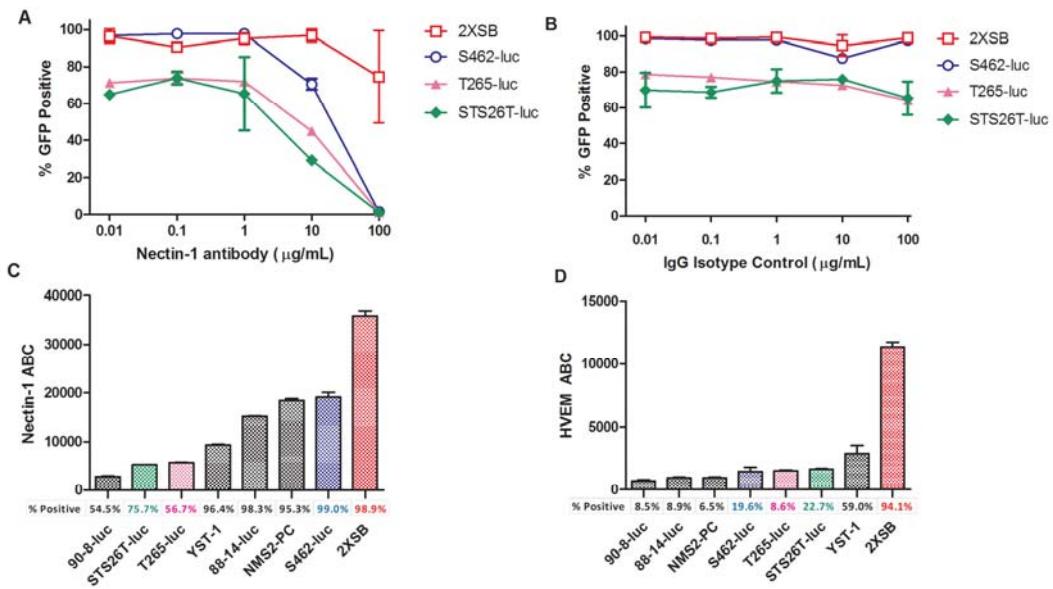
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Supplementary Information accompanies this paper on Gene Therapy website (<http://www.nature.com/gt>)

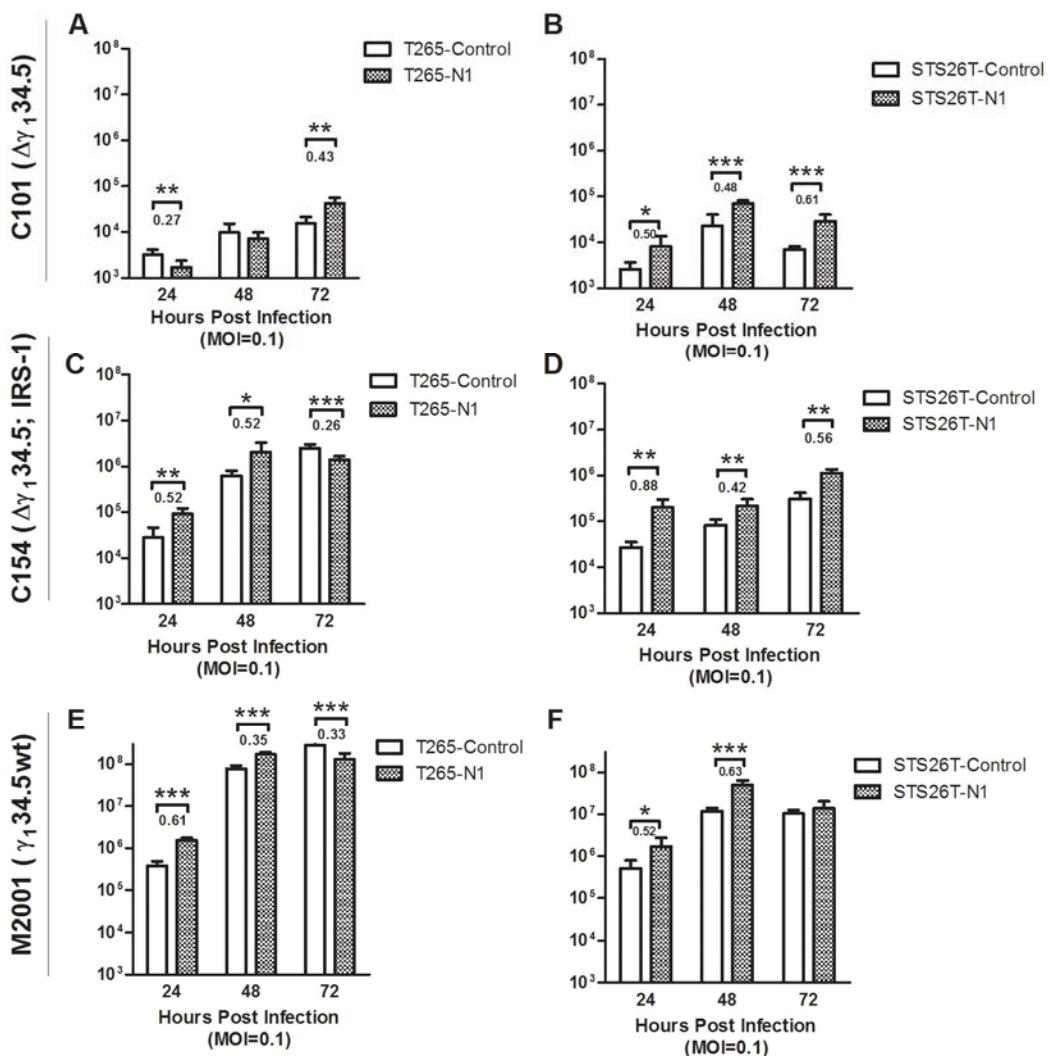


Supplemental Figure 1: Single-step viral recovery in MPNST cell lines. MPNST cell lines were subjected to single-step (MOI=10, 24 hr) infection by viruses M2001 (a), C134 (b), C101 (c), G207 (d), and R7020 (e) and the viral titers reported as the average total plaque forming units (PFU) as determined through limiting dilution plaque formation assay. Data were collected in triplicate and the standard deviation reported.



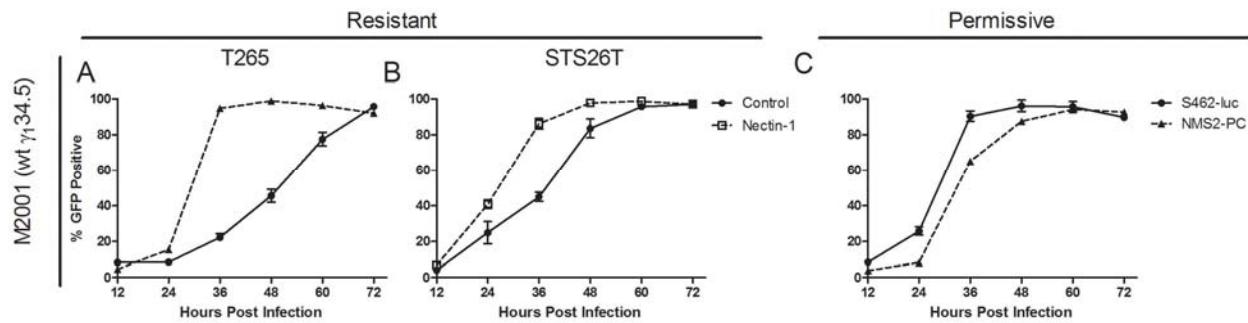
Supplemental Figure 2: Nectin-1 dependence of productive HSV-1 infection in resistant MPNST cell lines. MPNST cells were seeded  $3 \times 10^4$  cells per well in 96 well plates and allowed to adhere overnight. Nectin-1 antibody (clone R1.302, Biolegend) (A) or mouse IgG1 isotype control (Cell Signaling) (B) was diluted in growth media and incubated with cells at 4°C for 30

minutes. Cells were then infected with GFP expressing wt HSV-1 M2001 at an MOI of 5 and incubated at 37 °C for 20 hrs (S26T-luc, T265-luc, and S462-luc) or 12 hrs (2XSB). The earlier timepoint of analysis for 2XSB was necessary due to the rapid infection and deterioration of these cells at longer timepoints. Cells were trypsinized and resuspended in FACSs buffer and analyzed by flow cytometry for GFP expression. The % GFP positive is calculated as the percentage of viable cells which have a GFP intensity greater than an uninfected control. Measurements were taken in duplicate and the standard deviation reported. Nectin-1 (C) and HVEM (D) expression was quantified by flow cytometry after incubation with PE-conjugated mouse monoclonal antibody (Biolegend) with subsequent quantification using antibody quantification beads (Bangs Labs). The percentage of cells staining above the isotype control is also reported. Receptor quantification was performed in triplicate with the standard deviation reported.

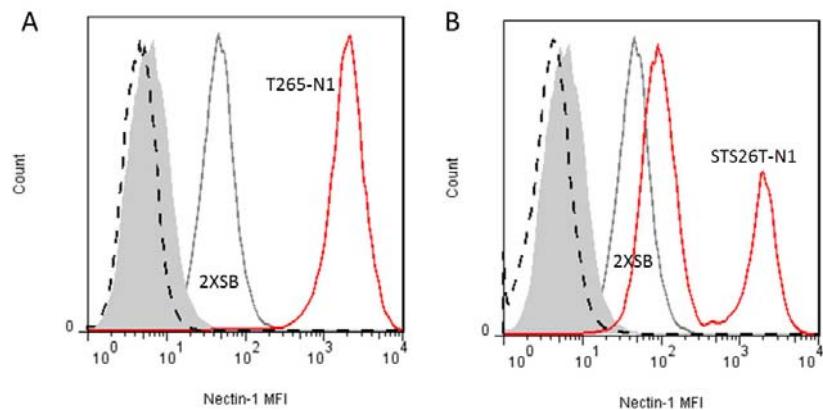


Supplemental Figure 3: Impact of increased nectin-1 expression on multi-step viral replication. Multi-step (MOI=0.1) replication assays using C101 (A,B), C154 (C,D), and M2001 (E,F)

viruses were performed in S26T-luc and T265-luc transduced with nectin-1 or control lentivirus. Samples were collected at the indicated timepoints following infection and titered using standard limiting dilution plaque formation assays. Data were collected in triplicate and the standard deviation reported. Comparison was made between nectin-1 overexpressing cells and control by 2-tailed Student's t-test. Significance was set at  $P < 0.05$ . For cells with significant changes in titer, the logarithm of the increase is reported below the significance marking.



Supplemental Figure 4: Impact of increased nectin-1 expression on wild-type HSV-1 spread in vitro. Resistant cell lines T265-luc (A) and STS26T-luc (B) and their nectin-1 transduced variants as well as permissive cell lines S462-luc and NMS2-PC (C) were infected in a multi-step assay (MOI=0.1) with GFP expressing wild-type HSV-1 M2001 and monitored over time for viral infection as evident by expression of viral GFP monitored by flow cytometry.



Supplemental Figure 5: Comparison of nectin-1 expression in 2XSB to nectin-1 overexpressing cell lines T265-N1 (A) and STS26T-N1 (B). Cell lines were analyzed by flow cytometry as described in the submitted manuscript. Dashed black or solid red lines respectively represent unlabeled or nectin-1 labeled resistant cell lines T265-N1 or STS26T-N1. Resistant cell lines in each graph are shown to express nectin-1 at an intensity greater than the highest natural nectin-1 expressing cell line 2XSB (gray line). Unlabeled 2XSB is also shown (shaded gray).